Dysbalanced BCR signaling in B cells of patients with systemic lupus erythematosus

D i s s e r t a t i o n

zur Erlangung des akademischen Grades

d o c t o r r e r u m n a t u r a l i u m

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Lebenswissenschaftlichen Fakultät

der Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: 11.09.2015

Zusammenfassung

Die systemische Autoimmunerkrankung Systemischer Lupus Erythematodes (SLE) ist durch den Verlust der Toleranz gegenüber Nukleinsäuren charakterisiert, was wiederum zu einer Produktion von Antikörpern (AK) gegen nukleare Antigene sowie anti-Doppelstrang DNA führen kann. Die Produktion dieser AK führt direkt bzw. via Immunkomplexe zu Gewebeschädigungen wie z.B. einer Lupus Nephritis. Aus diesem Grund spielen B-Zellen eine zentrale Rolle in der Pathogenese dieser Autoimmunerkrankung (1-3).

Bisher konnten Zusammenhänge zwischen der Entstehung von Autoimmunerkrankungen und einer veränderten B-Zellaktivierung hergestellt werden (4-6). In wie weit jedoch veränderte B-Zellrezeptor (BZR) Signalwege oder Co-Rezeptoren in diesem Prozess involviert sind, ist noch nicht ausreichend im humanen SLE untersucht worden.

Aus diesem Grund wurde in der vorliegenden Arbeit eine detaillierte Analyse des inhibitorischen Co-Rezeptors CD22, der Tyrosinkinase Syk und der Serinkinase Akt in B-Zellen des peripheren Blutes von SLE Patienten im Vergleich zu gesunden Kontrollen durchgeführt.

CD27- SLE B-Zellen zeigten eine erhöhte Expression und Aktivierung von CD22. Des Weiteren wurde in SLE CD27- als auch in CD27+ B-Zellen eine reduzierte Phosphorylierung von Syk und PLC-γ2 sowie ein reduzierter Calcium Influx nach BZR Aktivierung gemessen. Diese reduzierte BZR Aktivierung wurde unter anderem durch eine erhöhte Tyrosinphosphatase-Aktivität verursacht. Im Gegensatz zu den beiden Tyrosinkinasen Syk und PLCγ-2 zeigte die Serinkinase Akt eine erhöhte Phosphorylierung nach Stimulation in SLE im Vergleich zu gesunden B-Zellen. Dieses führte zu einem Ungleichgewicht zwischen dem Syk- und Akt Signalweg, welches wiederum das Überleben der Zelle nach BZR Stimulation in SLE Patienten begünstigte.

Während einer genaueren Untersuchung bezüglich der Expression von Syk in B-Zellen von SLE Patienten wurde eine Syk++ exprimierende CD27- B-Zellpopulation nachgewiesen. Die Frequenz dieser Population war im Vergleich zu gesunden Kontrollen signifikant erhöht, korrelierte jedoch nicht mit der Krankheitsaktivität. CD27-Syk++ B-Zellen zeigten eine mit CD27+ B-Zellen vergleichbare Syk- und Akt Phosphorylierung nach BZR Stimulation. Sie wurden als CD38-, CD19++, CD20++ und überwiegend CD21- mit einer reduzierten ABC-B1 Transporter Aktivität charakterisiert. Außerdem konnten

somatische Hypermutationen in den $V(D)J_H$ Genrearrangements, als auch eine Differenzierung zu IgG sekretierenden Plasmazellen nachgewiesen werden. Interessanter Weise konnte die *in vitro* Bildung von CD27-Syk++ B-Zellen durch die Stimulation mit INF-γ und TNF-α induziert werden.

Zusammenfassend zeigen die Daten, dass SLE Patienten eine Dysbalance in BZR abhängigen Signalwegen aufweisen, welche eine B-Zellsubpopulationen (CD27+, CD27-Syk++, CD27-Syk+) unabhängige Reduktion der p-Syk/p-Akt Ratio versursacht. Diese Verschiebung könnte zu einer defekten negativen Selektion und somit zur Bildung von autoreaktiven Zellen führen, die wiederum durch Überlebensvorteile persistieren könnten. Zusätzlich wurde im peripheren Blut von SLE Patienten eine bislang nicht bekannte CD27-Syk++ B-Zellpopulation nachgewiesen. Diese wies, trotz des fehlenden Gedächtnismarkers CD27, Gedächtnismerkmale auf und könnte für die bekannte erhöhte Plasmazell-induktion in SLE Patienten verantwortlich sein.

Somit konnte Syk als intrazellulärer Marker einer Gedächtnispopulation identifiziert werden. Des Weiteren stellt die Wiederherstellung der Balance von Syk- und Akt Phosphorylierung nach BZR Aktivierung einen erfolgsversprechenden Therapieansatz bei SLE Patienten dar, um die Entstehung und das Überleben von autoreaktiven B und Plasmazellen besser kontrollieren zu können.

Schlagwörter: Autoimmunität, SLE, B-Zelle, Autoreaktivität, B-Zellrezeptor Signalweiterleitung

Abstract

Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease in which loss of tolerance to nucleic acids results into the production of antibodies (Ab) against nuclear antigens (ANA) or double-stranded DNA (anti-dsDNA). This can lead to the formation of immune complexes which cause inflammation as well as tissue damages (1). Therefore, B cells might play a key role in the pathogenesis of this disease (2, 3). Several studies linked the development of autoimmunity to abnormalities of B cell receptor (BCR) associated signaling molecules (4-6). However, abnormalities of BCR associated co-receptors and downstream kinases with potential implications in selection processes are rare for human SLE. Thus, a comprehensive analysis of the inhibitory BCR co-receptor CD22, the spleen tyrosine kinase (Syk) and the pro-survival serine kinase Akt has been undertaken to gain new insights into potential BCR signaling disturbances in this autoimmune disease.

Here, CD27- SLE B cells showed an enhanced expression and activation of CD22. Furthermore, SLE CD27- and CD27+ B cells showed after BCR activation a diminished Syk and phospholipase C-γ2 (PLC-γ2) phosphorylation as well as a reduced calcium release compared to healthy donors (HD). This was related to an enhanced tyrosine phosphatase activity in these patients. In contrast, SLE B cells showed an increased phosphorylation of Akt after BCR activation and a disturbed balance between the intracellular Syk and Akt signaling pathway closely related to an enhanced survival of these cells.

Interestingly, analysis of the expression of Syk in SLE versus HD B cells revealed two different subsets within the CD20+CD27- population according to their expression of Syk (Syk++ and Syk+). The frequency of CD27-Syk++ B cells was significantly increased in SLE compared to HD; however the disease activity did not correlate with the frequency of this subset. Of note, CD27-Syk++ B cells showed a significant increased Syk and basal phosphorylated (p)-Syk expression as well as memory phosphorylation kinetics of Syk and Akt upon BCR engagement. Furthermore, CD27-Syk++ B cells were characterized as CD38, CD19++, CD20++ and mainly CD21- with reduced ABC-B1 transporter activity and exhibited somatically mutated IgV_H rearrangements. These cells shared characteristics of memory B cells and differentiated after *in vitro* stimulation with IL-10, IL-2, CpG and anti-IgM/IgG into IgG secreting plasma cells. Additionally, Syk++ B cells were inducible *in vitro* by the stimulation with IFN-γ, LPS or TNF-α.

This data indicate that B cells from SLE patients display an intrinsically disturbed balance of BCR related signaling pathways, resulting in a B cell subset (CD27+, CD27-Syk+, CD27-Syk++) independent reduced p-Syk/p-Akt ratio. This may lead to a diminished BCR dependent negative selection and enhanced survival of SLE B cells, permitting the emergence of autoreactive B and plasma cells. Furthermore, SLE patients exhibit an increased frequency of a novel CD27-Syk++ B cell subset with memory features, enhanced tonic BCR signaling and the capacity to differentiate in auto-Ab secreting cells.

The current study provides evidence that the use of intracellular markers, such as Syk, could permit a more precise delineation of CD27- memory B cell subsets in autoimmune diseases since the conventional used memory marker CD27 has some limitations. In addition, the balance between the BCR associated kinases Syk and Akt might be a promising therapeutic target to reduce the occurrence of autoreactive B and plasma cells.

Keywords: Autoimmunity, SLE, B cells, autoreactivity, B cell receptor signaling

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Abbreviation

abbreviation	meaning
%	percentage
°C	degree centigrade
Ab	antibody
ABC	ATP binding cassette
ACR	American College of Rheumatology
AID	activation induced cytidine deaminase
AK	Antikörper
ANA	anti-nuclear antibody
AP	alkaline phosphatase
APC	allophycocyanin
APCs	antigen presenting cell
APC-H7	allophycocyanin-H7
ASC	antibody secreting cell
BAFF	B cell activating factor
BCAP	B cell adaptor protein for PI3K
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
BD	Becton Dickinson
BILAG	British Isles Lupus Assessment Group
BLNK	B cell linker
BLK	B lymphocyte kinase
BM	bone marrow
BSA	bovines serum albumin
BTK	burton tyrosine kinase
BZR	B Zell Rezeptor
Ca^{2+}	calcium
Cbl	casitas B-lineage lymphoma
CD	cluster of differentiation
CD40L	CD40 Ligand
CLL	chronic lymphatic leukemia
CO_2	carbon dioxide
CpG ODN	cytosine-phosphate-guanine-oligonucleotide
CST	cytometer setup and tracking
D	diversity
DAS	disease activity score
DC	dendritic cell
DNA	deoxyribonucleic acid
ds	double-strand
EDTA	ethylendiamintetraacetate
ERK	extracellular signal-regulated kinase
FACS	fluorescent-activated cell sorting
FcγRIIb	gamma fragment crystallizable region receptor II b
FCS	fetal calf serum
FITC	fluorescein Isothiocyanate
FMO	fluorescence minus one-control
FO	follicular
FOXO-1	forkhead protein O-1
FSC	forward light scatter
g	relative centrifugal force
GC	germinal center
Grb2	growth factor receptor-bound protein 2
H	heavy Chain
h	hour
HD	healthy donor
HIV	human immunodeficiency patients
HRP	horseradish peroxidase

HSC	hematopoietic stem cell
IFN	interferon
Ig	immunoglobulin
IgA, IgD, IgE, IgG, IgM	immunoglobulin isotype A, D, E, G or M
IL	interleukin
IP3	inositoltriphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitor motif
J	joining
kDa	kilo Dalton
L	light chain
LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
ml	milliliter
MTO	MitoTracker orange
mTOR	mechanistic target of rapamycin
MZ	marginal zone
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NK	natural killer
nm	nanometer
ns	non significant
p	phospho
PacB	pac blue
PacO	pacific orange
PAMPs	pathogen associated molecular pattern
Pax	paired box protein
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy7	phycoerythrin cyanine 7
PerCp	peridinin chlorophyll-a protein
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PLC-γ2	phospholipase C-γ2
PMT	photomultiplier
Pro-B-cell	progenitor-B cell
PRR	pattern-recognition receptor
PS	phosphatidyl serine
pSS	primary Sjögren's syndrome
p-value	calculated probability
r	Pearson-correlation coefficient
RA	rheumatoid Arthritis
RNA	ribonucleic acid
RPMI	Rosswell Park Memorial Institute (place of origin)
RRX	rhodamine Red-X
RT	room temperature
RT	reverse transcriptase
S	serine
Sec	seconds
SHM	somatic hypermutation
sCD27	soluble CD27
SH2-Domänen	src-Homology 2
SHIP-1	signaling inositol polyphosphate phosphatase-1
SHP-1	src homology region 2 domain-containing phosphatase-1
Siglec	sialic acid-binding immunoglobulin-like lectin

SLE	Systemic Lupus Erythematosus
SLE	Systemischer Lupus Erythematodes
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLAM	Signaling lymphocytic activation molecule
ss	single stranded
SSC	sideward light scatter
Syk	spleen tyrosine kinase
trans	transitional
TD	T cell dependent
TGF	transformation growth factor
T_H	T helper
Thr	threonine
TI	T cell independent
TLR	toll-like receptors
TMB	3,3'5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
Tris	tris-aminomethane
V	variable
Y	tyrosine
µL	microliter
µM	micromole

1. Introduction

1.1 The immune system

The immune system is a complex system protecting an organism against diseases caused by pathogens, such as bacteria, viruses and parasites and can be divided into the innate and adaptive immune system (7). The innate immune system plays a crucial role during the first defense against pathogens in an antigen independent manner, recognizing different specific pathogen associated molecular patterns (PAMP). PAMPs bind pattern-recognition receptors (PRR) and induce a complex signaling cascade resulting in the activation of these cells (7). One important subclass of these PRRs are toll-like receptors (TLRs) (8). Ten TLRs are known in humans, each of them recognizing a specific PAMP. TLR9 for example binds dsDNA whereas RNA is recognized by TLR7 (8).

Besides anatomic barriers, including skin and mucous membranes, humoral factors like the complements system as well as cellular components (neutrophils, macrophages, dendritic cells (DC), and natural killer (NK) cells) are permanent present to immediately react within hours against pathogens (7).

The adaptive immune system (cellular immunity) or antigen specific immune response driven by lymphocytes mainly T cells (cell-mediated immunity) and B cells (humoral immunity) which derive from hematopoietic stem cells (HSC) in the bone marrow (BM) takes places 4-7 days after infection. This immune response leads to the production of antigen specific antibody (Ab) by B cells and - based on sufficient T cell help and co-stimulation - development of an immunological memory (7).

In addition to the defense against infections the discrimination between self and non-self is a crucial function of the immune system (9, 10). A breakdown of self tolerance can lead to the development of autoimmune diseases, such as systemic lupus erythematosus (SLE) as a prototypic systemic disorder (11-13).

1.2 B lymphocytes

B cells are indispensable during an immune response by their generation and secretion of highly specific Ab, long-lived immunological memory and their function as antigen presenting cells (APCs) as well as the establishment of germinal centers (GC;

dynamic microanatomical lymphoid structures), co-stimulation and the production of cytokines (7, 9).

1.2.1 B cell development

Early B cells are generated in the BM from HSC (CD34+CD38-CD10-CD19-). HSCs differentiate further either in myeloid or lymphoid stem cells. The myeloid stem cells are the progenitors for granulocytes, monocytes, macrophages and DC's. The lymphoid stem cells give rise to B, T and NK cells (14) (**Figure 1-1**).

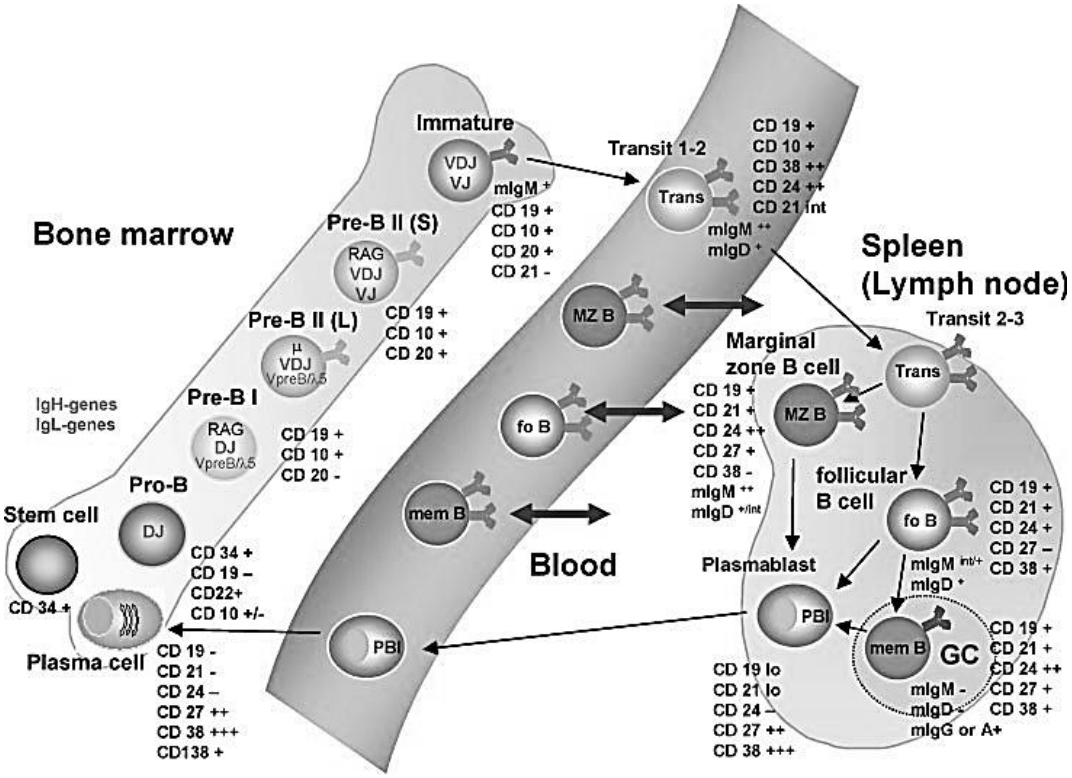

Figure 1-1: Schematic representation of the B cell development and their surface marker expression. B cells develop in the BM into immature B cells and migrate as transitional (transit) B cells into secondary lymphoid organs were they differentiate into marginal zone (MZ) or follicular (FO) B cells and develop further after antigen binding into memory and plasmablasts (PB)/plasma cells (15).

The development, maturation and selection of B cells are tightly regulated by the affinity and activation threshold of the B cell receptor (BCR) for self or foreign antigens (16, 17). This is controlled by a specialized microenvironment including soluble factors (cytokines like interleukin (IL)-7), transcription factors (e.g. paired box protein (Pax)-5) and other immune cells (e.g. stromal cells) (18). The differentiation of B cell precursors can be identified by the expression of specific genes, cluster of differentiations (CD's) and the rearrangement status of Immunoglobulin (Ig) heavy (H) and light (L) chains. Pro-B cells (CD34+CD10+CD19+IgM-) develop from early lymphoid progenitors which do not

express any functional Ig genes. The diversity, specificity and affinity of the BCR is achieved by a random H (V (variable)$_H$, D (diversity)$_H$, J (joining)$_H$) and L (V_L, J_L) chain rearrangement of the V(D)J_H chain locus which is regulated by the expression of the lymphocyte specific recombination enzymes RAG1, RAG2 and TdT (14). Pre-B cells (CD34-CD10+CD19+IgM-) start to express a pre-BCR (IgH chain and surrogate L chains). Signaling events through the pre-BCR induces the rearrangement as well as allelic exclusion and leads to the expression of a functional BCR composed by an L chain, H chain and Igα/β chain which is expressed on immature B cells (7, 14, 19). Immature B cells (CD34-CD10+CD19+IgM+) express membrane IgM, leave the BM as transitional B cells (CD34-CD19-CD27-CD24++CD38++) and migrate into secondary lymphoid organs (spleen, tonsil, lymph nodes) (14).

Transitional B cells which reside in the lymphoid tissue differentiate either into follicular (FO; CD19+CD20+CD21+CD23+CD24+IgM^{low}IgD+) or marginal zone (MZ) B cells (CD19+CD20+CD23+CD21++IgM+IgD^{low}) (14). The B cell fate decision is among others dependent on phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NFκ–B), B cell activating factor (BAFF) and the BCR affinity (20). It is assumed that CD22 may also be involved in the dichotomic decision between FO or MZ B cell differentiation (21). Furthermore, MZ B cells differentiate in short-lived IgM secreting plasma cells and are located between the red and white pulp of the spleen but has been also found in the peripheral blood (14). FO B cells reside in the follicle of the lymph node and spleen, are involved in T dependent (TD) immune responses and give rise to IgM and isotype switched memory B and plasma cells (14).

1.2.2 B cell activation and memory generation

The generation of memory B cell and long-lived secreting plasma cells out of naïve mature FO or MZ B cells is essential for the long-term humoral immunity, protection against pathogens and a rapid recall response against secondary infection (22-25). However, how the protective memory is maintained for more than 80 years is still under discussion especially how the different half life of IgG can be explained and how reactive B cell memory in the absence of the cognate antigens is maintained.

Mature naïve B cell recirculate through the bloodstream and migrate to secondary lymphoid organs where they encounter antigens and get fully activated (26). After antigen

encounter naïve B cells develop into memory B or plasma cells. Memory B cell subsets originate from distinct GC dependent or independent maturation pathways as well as T cell independent (TI) or TD activation and give rise to a heterogeneous memory population. The expression of the surface marker CD27, a member of the TNF receptor family has been established to identify antigen experienced memory B cells (27). CD27 is upregulated on antigen activated B cells, interacts with CD70 on activated T and B cells during GC reactions (28-31) and is important for B cell expansion, differentiation and Ab production. However, in the last years CD27 has been found to have certain limitations as universal marker for memory B cells in particular in patients with HIV, chronic infections and autoimmune diseases since a number of CD27- isotype-switched B cell population were found (32-35). For instance, SLE patients display increased frequencies of CD27-IgD-CD95+ (36) memory B cells with somatic hypermutation (SHM) of Ig rearrangements. Similar populations were reported in the tonsil and in the blood of human immunodeficiency virus (HIV) infected patients as well as in elderly subjects (32, 35, 37).

The expression of an inactive ATP-binding cassette (ABC)-B1 transporter and SHM in the Ig gene as well as a class-switched isotype are important characteristics to identify antigen experienced memory B cells in humans (24, 38). Recently, also the expression of FcRH4 and CD45RB has been described to be memory restricted (39, 40). However, a universal marker for all existing memory B cell subsets independent of their CD27 or isotype expression has not been identified yet.

1.2.3 T cell independent immune response

The antigen itself defines the type of the immune response. In the TD immune activation proteins bind to the BCR and require the help of T cells to induce a specific immune response. In contrast, large peptides or polysaccharides can stimulate B cells directly without additional T cell help. TI immune responses can be subdivided into TI-Type 1 (large polysaccharide) and TI-Type 2 (highly repetitious molecules) antigens. TI-Typ1 antigens require additional interaction with an innate receptor whereas TI-Type 2 antigens can directly activate B cells by extensive cross-linking of the BCR and induces a rapid early immune response against invading and mainly blood borne pathogens (41-43). The TI immune response consists largely of IgM Ab with low affinity. These Ab do not show significant H chain class-switch or affinity maturation. Only Typ-2 TI immune responses can induce an immunological memory (42, 43). Among others, MZ B cells can

be activated by blood born pathogens and differentiate via the TI pathway into CD27+IgM+IgD+ memory B cells. These cells play an important role during an immune response against encapsulated bacteria (44).

1.2.4 T cell dependent immune response

During the primary immune response antigen activated FO B cells interact with specific T cells to differentiate either into short-lived plasma cells, GC independent early memory B cells or in a GC dependent reaction into class-switched high affine long-lived memory B and plasma cells (45-47) (**Figure 1-2**).

Soluble or large particular antigens (greater than 70 kDa) are presented by APCs whereas small soluble antigens can directly pass by afferent lymph vessels and are independent by a cell-mediated antigen presentation. Internalization and processing of the antigen by B cells as well as the presentation on major histocompatibility complex (MHC) molecules-class II complexes towards cognate CD4+ T helper (T_H) cells is essential for the initiation of the GC reaction. To activate antigen specific B cells, three signals are necessary. First, the binding of the T cell receptor to the antigen presenting B cell. Second, the ligation of CD40 expressed on B cells by the CD40 ligand (co-stimulation). Third, the secretion of a specific cytokine profile (e.g. IL2, IL4, IL-5) (43, 46).

After activation, B cells migrate to the B-T cell border and upregulate MHC-class II molecules which promote a more efficiently cognate T cell help. They start to proliferate and differentiate into short-lived plasmablasts with mainly IgM low affinity Ab (extrafollicular reaction) or migrate into the B cell follicle. There they induce GC formation, SHM, class-switch (IgM to IgG, IgA, IgA or IgE), affinity maturation and differentiation into high affinity Ab producing plasma cells and/or long-lived memory B cells (43, 46).

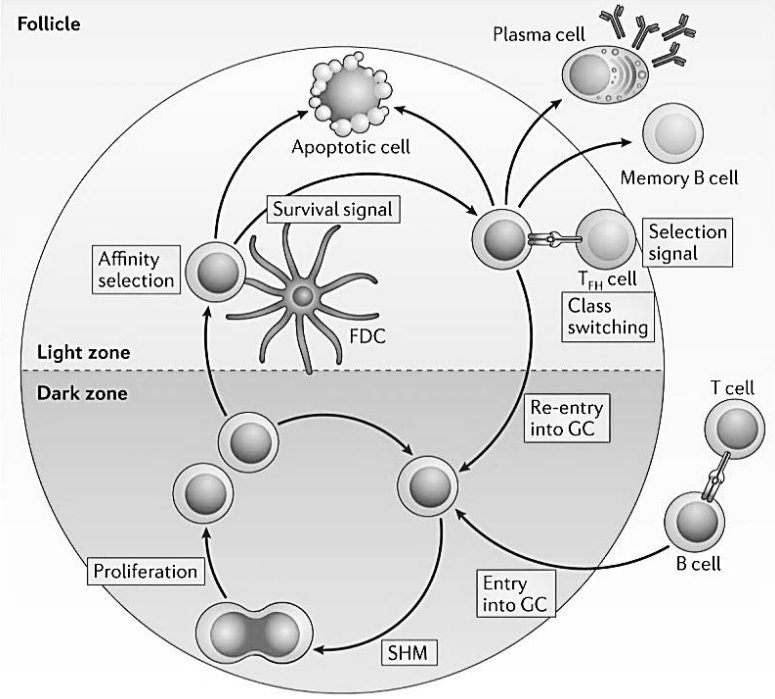

Figure 1-2: Schematic representation of the germinal center (GC) reaction. Activated B cells migrate into the dark zone where they undergo somatic hypermutation (SHM) and rapid proliferation. Afterwards they move into the light zone to get positive selected by follicular dendritic cells (FDC) and T cells. After positive selection the non autoreactive high affinity B cells develop into class-switched memory B or plasma cells whereas the autoreactive B cells undergo apoptosis or re-enter the GC reaction (48).

In the dark zone of the GC activated B cells (centroblasts) undergo proliferation and SHM. SHM are point mutations introduced by activating-induced cytidine deaminase (AID) in the V(D)J viable region gene segments which can lead to a change of the amino acid sequence and eventually to an increase of the BCR affinity against the specific pathogen. After this process, B cells migrate as centrocytes into the light zone where they undergo a stringent selection process controlled by antigen presenting follicular DCs and follicular T_H cells. The newly generated Ab mutants are selected dependent on their BCR affinity. B cells expressing a high affine BCR upregulate the expression of MHC-class II molecules which in turn lead to a stronger T cell activation and a better competition for the limited T cell help in the GC light zone. Positively selected cells start to differentiate into class-switched memory B cells whereas low affinity B cells undergo another round of proliferation and SHM or receive dead signals (43, 46, 49, 50).

1.2.5 Extrafollicular B cell activation

During an extrafollicular immune reaction, activated FO B cells differentiate into short-lived memory and Ab secreting plasma cells without entering the GC reaction. The choice of B cells to either differentiate rapidly into extrafollicular plasma cells or to undergo

GC reaction (hypermutation and affinity maturation) is dependent on the BCR affinity and antigen density. Low affinity or antigen density selectively favors GC reactions, whereas higher affinity induces a higher rate of extrafollicular responses (51).

In addition, it has been shown, that a strong CD40 stimulation can directly induce the development of memory B cells, mainly IgM+ but also CD27-IgA+ B cells, via the GC independent pathway and therefore favors an early memory B cell formation (45, 47). In line with this, B cells displaying large numbers of antigen-specific MHC-class II complexes can interact with large amounts of CD40 ligands on helper T cells leading to an extrafollicular B cell activation (45).

1.2.6 B cell tolerance

The SHM of Ig gene segments leads to a high diversity of the Ab repertoire against pathogens but may also give rise to self-reactive clones. In addition to GC selection process multiple tolerance mechanisms exist to control the emergence of autoreactive B cells and the development of autoimmunity (9, 52).

The first control of self-reactivity is the central tolerance checkpoint in the BM (central selection) (10). Deletion, receptor editing and anergy lead to the elimination of autoreactive B cells. During this selection processes the strength of the BCR response and the tonic or baseline BCR signaling against antigens play a crucial role and regulates the development and survival of B cells (16). In fact, an increased spleen tyrosine kinase (Syk) activity which leads to a hyperactive BCR response was sufficient to induce cell death of mouse lymphoblastic leukemia B cells indicating the importance of BCR associated signaling molecules (53). During the negative selection process, newly generated immature B cells that react with self-antigens are eliminated either by deletion (apoptosis) or receptor editing which induces an alternative L chain usage (54). Anergy is an additional mechanism to in-activate autoreactive B cell clones. It is characterized by unresponsiveness against antigens induced by the up regulation of inhibitory BCR co-receptors which in turns increases the activation threshold (14). Non autoreactive immature B cells continue to differentiate and get positively selected before they can leave the BM. Immature B cells have to reach a sufficient signaling level to get positive selected and to initiate further differentiation into the transitional B cell stage (10, 55). A hyperresponsive as well as a non-responsive phenotype against an antigen leads to the

elimination of this B cell clone. Thus, an intermediate BCR response is necessary for proper selection and differentiation into memory B and plasma cells (10, 55).

Self-reactive B cells which escapes the central tolerance in the BM and are autoreactive against tissue specific antigens which are not present in the BM gets selected by additional peripheral tolerance mechanisms. Activated B cells need co-stimulatory signals provided by CD4+ T_H cells. In addition, intrinsically regulation mechanisms which involves kinases and phosphatases to modulate the strength of the BCR signal and determines the B cell fate are necessary for proper selection (16, 56). During the GC reaction another selection of self-reactive B cell clones occur (peripheral tolerance). Low affinity B cells which lack adequate FDC and CD4+ follicular T cell help undergo apoptosis or another round of SHM (positive selection). Also in the late memory or plasma cell stage different mechanisms exist to circumvent self-reactivity. How this mechanisms works is still not clear but the blockade of differentiation into memory and plasma cells, the inhibition of Ab production and a limited survival within niches are possible mechanisms (9).

A breakdown of self-tolerance by defects in selection processes can lead to autoimmune diseases as it has been described for SLE (11-13). It has been published that defects in the BCR signaling may play an essential role during the development, selection and maintenance of autoimmunity (11, 53, 57-59). However, to which extend BCR associated receptors and its signaling molecules as well as the balance between kinases and phosphatases are involved in human autoimmune diseases are still rare.

1.3 B cell receptor structure and signaling

1.3.1 Organization of the surface BCR complex

The antigen receptor expressed on B cells is a transmembrane protein complex, the membrane-bound form of an Ig. It is assembled of different subunits including an IgH chain, covalently linked with disulfide-bridges to an IgL chain. The antigen binding site is located in the variable region of the amino-terminal of the IgH and IgL chain. The diversity and specificity of the antigen binding site is generated by V(D)J rearrangements and subsequently tuned by SHM. The carboxy-terminal region of the BCR determines the Isotype (IgM, IgD, IgG, IgA and IgE) (60). Surface IgM and IgD is expressed mainly on immature and mature naïve B cells (pre-switch), whereas IgG+, IgA+ and IgE+ post-switch B cells are generated after B cell activation and isotype class-switching (60).

In contrast to the soluble form of the Ig secreted by terminal differentiated plasma cells, the membrane-bound BCR is associated with two heterodimer polypeptides (Igα and Igβ). The intracellular part of Igα and Igβ contains immunoreceptor tyrosine-based activation motifs (ITAMs) to initiate the B cell response and determine the B cell fate decision (60).

1.3.2 B cell signaling

Binding of antigens to the BCR induces an affinity dependent clustering of certain molecules. High affine BCRs show rapid microcluster formation and enhanced recruitment of BCR key kinases as well as an enhanced calcium (Ca^{2+}) influx (61). This microcluster formation leads to an activation of a signaling cascade with the induction of a specific gene expression profile regulating proliferation, survival and apoptosis of activated B cells (17, 61). Besides multivalent and soluble antigens, also monovalent antigens are sufficient to induce the initiation of BCR capping, internalization of the antigen bound BCR and the presentation on MHC-class II molecules to CD4+ T_H cells (60).

During the initiation of the BCR clustering, the membrane topology and lipid raft composition plays an essential role. In resting B cells the BCR is excluded from the lipid rafts. After activation, the BCR moves into the lipid raft areas and is forced to open the cytoplasmic domain to get activated by Lyn (56, 62-64). Lyn activates the ITAM motifs within the cytoplasmic Igα/β (CD79a/b) tail of the BCR which directly interact with the signaling machinery to promote the recruitment of kinases. Lyn plays an essential role as negative and positive regulator of BCR activation (65, 66). Phosphorylation of Igα/β by Lyn leads to the recruitment of the SH2-containing kinase Syk which is recruited to the activated microcluster and translates the extracellular engagement into an intracellular signal in an orchestrated manner (63, 67).

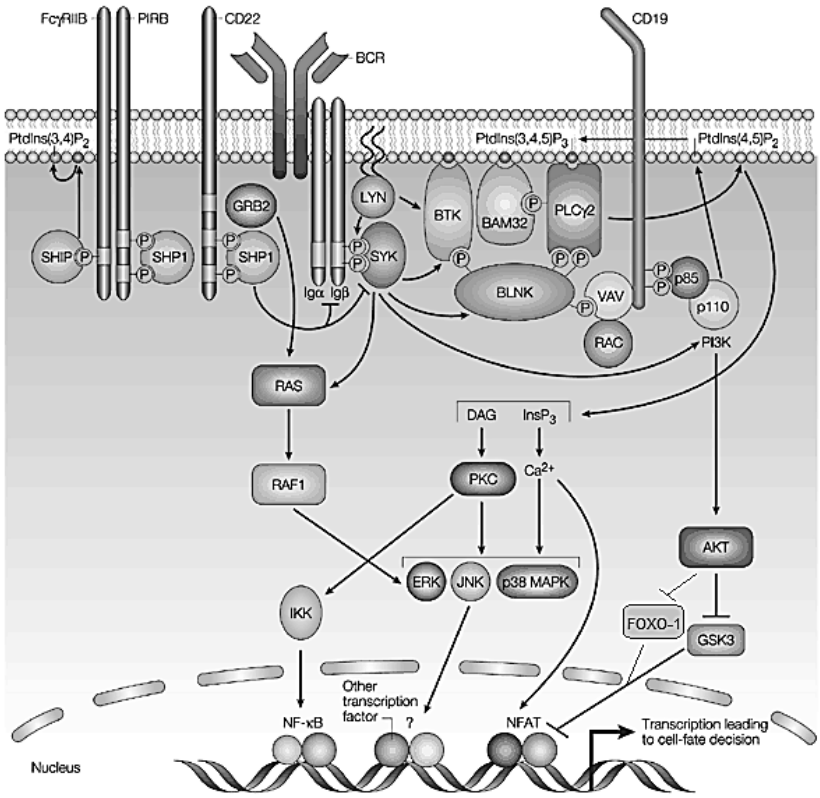

Figure 1-3: Schematic diagram of the central BCR signaling cascade. After antigen binding, the intracellular tail of the BCR becomes activated by Lyn leading to the recruitment of Syk and further downstream to the influx of Ca^{2+} and the activation of the transcription factors NFκ-B and NFAT. Lyn also activates BTK which induces the activation of CD19 and the PI3K/Akt survival pathway leading to an inhibition of the transcription factors FOXO-1 and GSK3. Negative regulation of the BCR response occurs by the activation of CD22 and FcγRIIb. CD22 activation recruits SHP-1 which in turn dephosphorylates Syk and dampens the BCR signal [modified according to (56)].

Subsequently, signaling molecules such as bruton's tyrosine kinase (Btk) and phospholipase C-γ2 (PLC-γ2) accumulate in a signalosome to initiate internalization, antigen processing, 1,4,5-trisphosphate (IP3) generation and Ca^{2+} release. This lead among others to the activation of the transcription factors nuclear factor of activated T cells (NF-AT) and nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NFκ-B) (68-71) inducing a specific gene expression profile. Lyn also activates B cell adaptor proteins (BCAP and BLNK) in a CD19-dependent manner resulting in the activation of the PI3K/Akt pathway which has been shown to play an important role in cell survival by regulating forkhead box protein (FOX) O1 (72-74) (**Figure 1-3**).

BCR signaling differs between naïve and memory B cells and is dependent on the isotype expressed on the B cell surface. Compared to naïve, memory B cells show a stable BCR response, a stronger formation of BCR oligomers, microcluster formation and a more effective recruitment of kinases. Memory B cells also show higher phosphorylation kinetics compared to naïve B cells suggesting a lower activation threshold to respond

faster during secondary immune responses (75, 76). Intrinsic differences, such as the expression of genes involved in activation, co-stimulation and survival are responsible for the efficient BCR response in memory B cells (77).

The BCR isotype has an impact on the signaling outcome since a different BCR response has been reported in IgM+ compared to IgG+ memory B cells. The density of membrane-bound Ig is similar between IgG+ and IgM+ memory B cells, the level of p-Syk, p-PI3K, p-extracellular signal-regulated kinase (ERK) and p-p38 recruited to the membrane is significantly higher in IgG+ compared to IgM+ B cells (75).

The antigen inducing BCR signaling is not the only essential factor during B cell development, tonic or basal ligand independent signaling via the BCR is also important for the survival and maintenance as well as the selection of B cells. Even without antigen binding, BCR oligomers are formed to provide a constant low-level of B cell activation that appear to be critical for the overall B cell survival (17, 78).

Besides the activation and accumulation of intracellular kinases, the recruitment and balance of positive (CD19, CD45 and CD21) and inhibitory co-receptors (CD22 and FcγRIIb) are important to ensure sufficient BCR signaling and peripheral B cell tolerance (40, 79-82). Imbalances between these positive and negative BCR co-receptors can lead to autoimmunity as it has been reported for human SLE (83-85). How this dysbalance influences the signaling outcome and therefore the B cell fate is still not fully understood.

1.3.2.1 Spleen tyrosine kinase (Syk) and its function in B cells

The non-receptor cytoplasmic tyrosine kinase Syk which is expressed in all hematopoietic- and non-hematopoietic cells like platelets, fibroblasts and endothelial cells plays a crucial role during the adaptive immune receptor signaling response. Other biological functions are cellular adhesion, innate immune responses and vascular development (86, 87). Syk is a 72 kDa enzyme containing two SCR homology 2 (SH2) domains separated by a linker and a kinase domain. The kinase domain is inactive in the resting state due to an autoinhibitory conformation. The kinase becomes activated by the translocation to the plasma membrane and binding of the SH2 domains to the activated BCR complex. This activation leads to a change in its conformation which permits the phosphorylation of tyrosine (Y) residues within the linker region and the C-terminal tail (Y131, Y323, Y348, Y352, and Y629-631). This phosphorylation activates the initiator

function of Syk and leads to an amplification of the BCR signal by a rapid phosphorylation of the neighboring ITAM sequences (87).

The residue Y131 is responsible for the dissociation of Syk from the phosphorylated ITAMs of the BCR complex and is necessary to switch off Syk activity. Binding of the E3 ubiquitin ligase casitas B-lineage lymphoma (Cbl) to Syk is initiated by the phosphorylation of Y323 and therefore important for its degradation. The tyrosine residues Y348 and Y352 are two early phosphorylation sites of Syk that lead to the recruitment and initiation of further downstream signaling cascades and signalosome formation (88). Syk activates CD19, the adapter protein BCAP as well as the PI3K pathway, B cell linker protein (BLNK), B lymphocyte kinase (BLK), Vav and PLC-γ2 leading to an intracellular Ca^{2+} release, proliferation, survival and differentiation. However, a Syk independent CD19/PI3K/Akt activation pathway has been identified leading to B cell survival even in the absence of this kinase (89). Syk plays an indispensable role during BCR signaling, maintenance of mature B cells, ITAM mediated tonic signaling and B cell development in the BM (17, 53, 87).

One negative regulator of Syk is the phosphatase Src homology region 2 domain-containing phosphatase-1 (SHP-1). It becomes activated after BCR engagement by the inhibitory co-receptor CD22 to prevent chronic- or hyperactivation (90). A second negative regulator of Syk is Cbl. Cbl leads to the ubiquitylation and hereafter to the degradation of Syk (91-93). Deficiency of Syk leads to a complete absence of mature B cells and is important during the B cell transition of pro- into pre-B cells and FO into GC B cells as well as during positive selection processes of immature B cells (59, 94). Abnormalities of Syk have been described to be involved in several forms of leukemia's (95, 96), T cell lymphoma (97) and autoimmune diseases like SLE (98, 99). Thus, the regulation of Syk by specific inhibitors, such as R406, seems to be an attractive therapeutic possibility (100, 101).

Nevertheless, human studies on the expression of Syk in B cells and its possible relation to SLE immunopathogenesis are rare (102) and need to be further evaluated.

1.3.2.2 Functional role of the serine kinase Akt in B cells

The serine (S)/threonine (Thr) kinase Akt is known to be involved in B cell survival and cell cycle progression. The activation of Akt is initiated in a Lyn dependent manner by PI3K after BCR and CD19 activation (74). Three isoforms of Akt are expressed in

B cells and are constituted of an N-terminal pleckstrin homology containing domain, a threonine residue (Thr308) in the activation loop and a hydrophobic motif (S473). Both activation sites need to be phosphorylated to fully activate Akt. One important target of Akt is the transcription factor FOXO-1. Its phosphorylation leads to the nuclear export and degradation and therefore suppression of its transcriptional activity (74).

The activation of Akt by CD19/PI3K and the capability of Akt to inactivate FOXO-1 factors regulate the development of mature into MZ B cells. Mice lacking CD19 show a loss of MZ B cells (103, 104). This data indicate that the PI3K/Akt signaling pathway induces the formation of short-lived, mainly IgM+ plasma cells generated during TI immune responses. The choice between rapid extrafollicular plasma cell differentiation and GC reaction seem to be dependent on the strength of the PI3K/Akt signaling pathway and therefore by the activity of FOXO-1 (103, 105). Loss of the phosphatase and Tensin homolog (PTEN) led to an increased differentiation into Ab secreting plasma cells and a reduction in GC dependent class-switch recombination by elevated PI3K/Akt activation (74).

However, detailed studies on the activation kinetics of Akt and the interaction of Akt and Syk dependent signaling pathways in human SLE B cells are scarce.

1.3.2.3 BCR co-receptor assembly and functional consequences

BCR associated co-receptors modulate the signaling threshold and are crucial during B cell development and cellular selection processes (16, 17).

On the one hand, an important negative regulator of the BCR signaling is the sialic acid-binding lectin CD22 which is exclusively expressed on B cells (79). During B cell development CD22 is upregulated and reaches its maximum at the naïve B cell stage. After B cell activation CD22 is downregulated on memory B cells and absence on plasma cells (106). CD22 negatively regulates the BCR signaling by recruiting several signaling molecules to the cell membrane after BCR activation, including Lyn, Syk, growth factor receptor-bound protein (Grb) 2, signaling inositol polyphosphate phosphatase (SHIP), PI3K and PLC-γ2 (107-110). Key molecules of this downstream signaling cascade are the tyrosine phosphatase SHP-1 and Syk. Due to the wide range of CD22 ligands, the exact mechanism of the inhibitory activity of CD22 has not been fully revealed yet (79, 107). Recent studies indicate that CD22 can bind specific to α2-6-sialylated glycoproteins on the cell surface in *cis* (same cell) and *trans* (other cell), which seems to play an

important role for its inhibitory properties. Furthermore, CD22 regulates cell adhesion and migration of B cells (106). After BCR activation Lyn initiates Syk recruitment as well as the phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic tail of CD22. One important phosphorylation site is Y822 which is responsible for the recruitment and activation of the phosphatase SHP-1 leading to a dephosphorylation of Syk as it has been shown for human SLE B cells pre-treated with the monoclonal anti-CD22 Ab epratuzumab (111). CD22 deficient mice show a hyperresponsive Ca^{2+} response as well as increased levels of auto-Ab (112).

On the other hand, positive regulators like the co-receptor CD19 influence the strength of signals transduced through the BCR by lowering the signaling threshold. CD19 does not only function as a BCR co-receptor, it can also be used as a lineage marker for B cells and is expressed in conjunction with CD21 and CD81 (80, 113). Upon antigen binding to the BCR, the cytoplasmic tail of CD19 gets activated and induces the activation of the PI3K/Akt survival pathway. Indeed CD19/CD21 co-receptor signaling has been implicated to regulate B cell survival during primary immune responses (72, 80). Mice lacking CD19 show defects in Ab secretion, GC formation and affinity maturation during TD immune responses (114).

Thus, modulating the BCR response is more complex. To fine tune the BCR signal and prevent autoimmunity, an equilibrium of positive and negative regulating co-receptors and their associated kinases and phosphatases is required (84, 85, 115, 116).

1.4 Autoimmune diseases and loss of B cell tolerance

The breakdown of tolerance against self-antigens and therefore the generation of autoreactive B and plasma cells lead to the development of autoimmunity. Autoimmune diseases can be organ-specific, such as multiple sclerosis (nerve cells), diabetes mellitus Type 1 (insulin-producing cells) or systemic, such as vasculitides, SLE or various other diseases (60).

1.4.1 Systemic lupus erythematosus

SLE is a prototypic autoimmune disease in which loss of B cell tolerance is reflected by the production of anti-nuclear antibodies (ANA) including Ab against double stranded DNA (dsDNA) due to defects in the clearance of dying cells (117-119). The subsequent formation of immune complexes can result in inflammation and tissue

damage as well as an aberrant production of cytokines by cells of the adaptive and innate immune system including IL-6, IL-10, tumor necrosis factor-α (TNF-α), Interferon-γ (INF-γ), IL-21, IL-2, IL-4 and BAFF (1, 57, 120-130). T cells for example are the major source of IL-4, IL-17 and INF- γ but strikingly reduced IL-2 in the peripheral blood of SLE patients, whereas IL-6 and IL-10 was mainly produced by monocytes (131). B cell lymphopenia and alteration in peripheral B cell subsets are another common feature of SLE patients (3). Several studies reported dysregulation of B cell homeostasis in these patients, including an increased frequency of transitional type 1 (T1) and pre-naïve B cells (132, 133), $CD27^{high}$ or $HLA\text{-}DR^{high}CD27^{high}$ plasmablasts (134, 135), $CD27+/\text{-}CD19^{high}$ (136, 137) and $CD27+/\text{-}CD21^{low/neg}$ (138, 139) as well as CD27-IgD- (32) and CD27-IgD-CD95+ (36) B cells. A central role in the pathogenesis of SLE plays B cell hyperactivity shown by an increased number of circulating plasma cells (135, 140), spontaneous auto-Ab production *in vitro* (141, 142), global increased tyrosine phosphorylation after BCR stimulation (143) and the occurrence of hypergammaglobulinemia (144). The disease activity of SLE patients is assessed by using clinical and laboratory markers and is represented by the systemic lupus erythematosus disease activity index (SLEDAI) or the British Isles lupus assessment grouped (BILAG) score (145). However, this ranking system has limitations and does not represent the clinical complexity of this disease.

The development of SLE is still not fully clarified but genetic predispositions (146-148), increased expression of IFN-related genes (149, 150) as well as intrinsic abnormalities related to signaling disturbances (4-6, 98, 151-154) have been found to play a mature role in B and T cells.

1.4.2 B cell tolerance and signaling in SLE

So far, there is no clear understanding to which extent defects in central (mainly related to autoreactive naïve B cells) or peripheral (mainly related to autoreactive memory B cells) tolerance are involved in the emergence of lupus autoimmunity. Defects in early tolerance checkpoints in SLE B cells (13) and the persistence of autoreactive naïve B cells even during SLE remission (155) have been described consistent with intrinsic disturbances of central selection (11, 12). In the study of Cappione et al., human SLE patients fail to exclude autoreactive B cells from GC reactions. The same study showed, that autoreactive B cell display a reduced Ca^{2+} influx after IgM activation (156).

Another body of evidence mainly based on the detection of mutated BCR rearrangements and phenotypic analyses suggests that most autoreactive B cells in SLE carry antigen-experienced BCR gene rearrangements as a result of T cell activation (57, 134, 157) suggesting defects of peripheral selection. However, central and peripheral tolerance mechanisms are strongly dependent on the strength of the BCR signal (53-55). Aberration in BCR induced signaling events can lead to the loss of tolerance, the development as well as maintenance of autoreactive B cells. Confirming this assumption, Lyn deficiency (158), $Lyn^{up/up}$ (159), Btk transgenic mice (58) as well as B cell specific insufficiency of SHIP-1 and PTEN (160, 161) led to an lupus-like phenotype in mice. A multi-signaling analysis of different BCR related signaling molecules revealed in different lupus mice models an hyperexpression and activation of the PI3K/Akt/mTOR, the MEK1/Erk1/2 signaling pathway together with p38, NFκ-B, multiple Bcl-2 members and cell-cycle molecules (162). Abnormalities of BCR signaling in human SLE have been described, such as a decreased Lyn (163), aberrant SHP-1 and CD45 expression leading to a disturbed lipid raft formation in B cells (5, 164). A reduced expression of PTEN which results in enhanced Akt activation (165) and a globally increased tyrosine phosphorylation (143) as well as an hyperresponsive BCR activation after IgM stimulation in naïve B cells (6) were also linked to B cell abnormalities in SLE patients. Additionally, two genetic polymorphisms in the transmembrane domain of the inhibitory co-receptor FcγRIIb have been associated with SLE. This polymorphisms led to reduced lipid raft localization and inhibition of the BCR signaling (166, 167). Other groups showed a reduced inhibitory effect of FcγRIIb on the BCR signaling which was due to an decreased expression of the phosphatase SHIP-1 (151) and a reduced expression level of FcγRIIb on CD27+ SLE B cells (168).

Nevertheless, also a diminished BCR response has been associated with SLE and other diseases. Especially gene mutations or overexpression as shown for chronic lymphatic leukemia (CLL) B cells of the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) which participates in the BCR signaling has been associated to an increased frequency of autoreactive immature B cells. These aberrations led to a block of BCR related signaling pathways that regulate apoptosis but induced enhanced BCR signaling that activates the pro-survival kinase Akt. These disturbances can lead to the circumvention of negative selection processes and the survival of autoreactive B cells (147, 169-171). Similar observations were made for SLE (172, 173). Evidence for a

diminished BCR response in autoimmunity was also provided by a genetic defect in the BCR related kinase BLK which led to a reduced BCR response and enhanced frequency of autoreactive naïve B cells (174). Also in mice models a rather diminished BCR response was predicted to be involved in SLE (175). In humans, SLE associated abnormalities of the BCR complex were found, including reduced levels of CD21 on certain SLE B cell subsets (138). B cells lacking CD21 are enriched in autoreactive and unresponsive B cell clones (176).

Thus, analyzing the expression and function of the BCR-related kinases could provide new insights to which extent signaling molecules contributes to SLE immunopathogenesis and the maintenance of autoreactivity.

2. Research aim

The strength of the B cell response plays a crucial role during B cell development, tolerance and cell fate decision. Defects in BCR related signaling pathways have been connected with the breakdown of self tolerance and the development of autoreactive memory B and antibody secreting plasma cells. Especially the balance between positive and negative co-receptors as well as phosphatases and kinases is crucial for proper antigen induced BCR signaling to prevent the emergence and maintenance of autoimmunity. Besides intrinsic abnormalities, SLE has been associated with an aberrant B cell homeostasis leading to the occurrence of abnormal B cell subsets. Especially within the memory B cell compartment abnormalities participating in the development and maintenance of this disease has been observed.

The aim of this study was to gain new insights into potential aberrations of intracellular BCR signaling disturbances that could lead to the development and maintenance of autoreactive B and plasma cells and the circumvention of tolerance checkpoints in patients with SLE. Analyzing SLE B cells more in detail will improve the understanding of SLE specific memory subsets regarding their contribution during pathogenic immune responses.

Two main questions were placed:

1.) **How is B cell tolerance in SLE patients disturbed and how can autoreactive clones survive and maintain in the repertoire of this disease?**
2.) **To which extent are abnormalities of B cell subsets, intracellular BCR signaling pathways and the BCR complex organization involved in this process?**

A comprehensive analysis in SLE patients versus controls regarding their expression, activation and localization of the BCR and its inhibitory co-receptor CD22 as well as its downstream kinases with a main focus on Syk versus Akt was performed.

3. Material and methods

3.1 Material

3.1.2 Chemicals and reagents

Table 3-1: Reagents

Reagents	Manufacturer
0.4% Trypan blue solution	Sigma-Aldrich, Saint Louis, USA
1-Step Turbo TMB-ELISA	Thermo Fisher Scientific Inc., Rockford, USA
Tris ($C_4H_{11}NO_3$)	Carl Roth, Karlsruhe, Germany
3-Amino-9-Ethylcarbazole (AEC) tablets	Sigma-Aldrich, Saint Louis, USA
4',6-diamidino-2-phenylindole (DAPI)	Roche, Basel, Switzerland
Acetic acid (CH_3COOH)	Carl Roth, Karlsruhe, Germany
Avian Myeloblastosis Virus RT	Sanquin Reagents, Amsterdam, Netherlands
Boric acid (H_3BO_3)	Merck KGaA, Darmstadt, Germany
BSA	Biomol, Hamburg, Germany
Cytometer setup and tracking beads	BD Biosciences GmbH, Plymouth, USA
Deoxynucleotides (dNTPs)	Sigma-Aldrich, Saint Louis, USA
Dimethyl sulfoxide (DMSO)	SERVA, Heidelberg, Germany
Dithiothreitol (DTT)	Sanquin Reagents, Amsterdam, Netherlands
Electrophoresis marker ml-100kbp+ DNA	Metabion international AG, Planegg, Germany
Ethanol 96%	Merck KGaA, Darmstadt, Germany
Ethanolamine	Merck KGaA, Darmstadt, Germany
EDTA 0.5 mM	Invitrogen Corporation., Camarillo, USA
FCS	DRFZ, Berlin, Germany
GelPilot DNA Loading Dye, 5x	Qiagen, Hilden, Germany
GelRed Nucleic Acid Gel Stain, 10,000X in water	Biotium, Hayward, CA, USA
Glycerol	Sigma-Aldrich, Saint Louis, USA
hydrochloric acid (HCl)	Merck, Darmstadt, Germany
hydrogen peroxide (H_2O_2)	Sigma-Aldrich, Saint Louis, USA
Indo-1 acetoxymethyl (AM) dye	Invitrogen Corporation., Camarillo, USA
LE Agarose	Biozym, Hessisch Oldendorf, Germany
Lymphocyte Separation Medium LSM 1077	PAA Laboratories, Paschin, Austria
Lyse/Fix-Puffer 5x PhosphoFlow	BD Biosciences GmbH, Plymouth, USA
Mito Tracker Orange CMTMRos	Life Technologies, Carlsbad, USA
p(dT)15 Primer	Roche Diagnostics GmbH, Berlin, Germany
PBS 1x	Biochrom, Berlin, Germany
PBS/BSA 0.5%	DRFZ, Berlin, Germany
Perm Buffer II PhosphoFlow	BD Biosciences GmbH, Plymouth, USA
Perm Buffer III PhosphoFlow	BD Biosciences GmbH, Plymouth, USA
Pierce Lysis IP buffer	Thermo Fisher Scientific Inc., Rockford, USA
p-nitrophenyl phosphate (p-NPP) tablets	Sigma-Aldrich, Saint Louis, USA
Protease inhibitor cocktail	Cell signaling technology, Cambridge, England
RNasin Plus RNase inhibitor	Promega Corporation, WI, USA
RPMI 1640 + GlutaMAX	Invitrogen Corporation., Camarillo, USA
RPMI 1640 + GlutaMAX+10 % FCS, 2-Mercaptoethanol (0.05 mM), Penicillin (100 U/ml) and Streptomycin (100 g/ml)	DRFZ, Berlin, Germany
Sodium acetate trihydrate ($NaOOC\text{-}CH_3 * 3\ H_2O$)	Sigma-Aldrich, Saint Louis, USA
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich, Saint Louis, USA
Sodium chloride (NaCl)	Merck KGaA, Darmstadt, Germany
Sodium fluoride (NaF)	Sigma-Aldrich, Saint Louis, USA
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich, Saint Louis, USA
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Saint Louis, USA
Spermidin	DRFZ, Berlin, Germany
Sphero Rainbow calibration particles 8 peaks	BD Biosciences GmbH, Plymouth, USA
β-Mercapthoethanol	Sigma-Aldrich, Saint Louis, USA

Sulfuric acid (H_2SO_4)	Carl Roth, Karlsruhe, Germany
Tetanus/diphtheria toxoid	Sanofi Pasteur, MSD GmbH, Leimen, Germany
TruCount-Lysis buffer 10x	BD Biosciences GmbH, Plymouth, USA
Tween 20	Sigma-Aldrich, Saint Louis, USA
Ultra pure Water	Biochrom, Berlin, Germany
Vectashield Hard set mounting medium with DAPI	Vector Laboratories, Burlingame, CA, USA
Goat F(ab`)$_2$ anti human IgM/IgG (H+L)	Jackson ImmunoResearch, Suffolk, UK
Human recombinant BAFF	Proptech, Hamburg, Germany
Human recombinant IFN-γ	Proptech, Hamburg, Germany
Human recombinant IL-10	Proptech, Hamburg, Germany
Human recombinant IL-2	Proptech, Hamburg, Germany
Human recombinant IL-21	Proptech, Hamburg, Germany
Human recombinant IL-6	Proptech, Hamburg, Germany
Human recombinant TNF-α	Proptech, Hamburg, Germany
CpG-B oligodeoxynucleotides 2006	Invitrogen Corporation., Camarillo, USA
LPS from Escherichia coli 055:B5	InvivoGen, San Diego, USA

3.1.2 Consumables

Table 3-2: Consumables

Consumables	Manufacturer
1.5 ml reaction tubes	Eppendorf AG, Hamburg, Germany
2.0 ml reaction tubes	Eppendorf AG, Hamburg, Germany
10 ml EDTA-tubes	BD Biosciences GmbH, Plymouth, USA
2 ml heparin-tubes	BD Biosciences GmbH, Plymouth, USA
5 ml FACS tubes 75x12mm	Sarstedt, Nümbrecht, Germany
CellStar 96-well culture plate, U-bottom	Greiner Bio-one, Frickenhausen, Germany
Falcon tube 15 and 50 ml	Sarstedt, Nümbrecht, Germany
Filter Pipette tips (1250 µl, 0.1-10 µl and 2-200 µl)	Sarstedt, Nümbrecht, Germany
MACS® cell separation columns	Miltenyi Biotec GmbH, Gladbach, Germany
Microscope cover glasses 24x50mm	VWR International, Radnor, USA
Microscope slides superfrost plus 25x75x1mm	Thermo Fisher Scientific Inc., Rockford, USA
MSIPN4550 Multiscreen-IP, 0,45 µm 96-well plates	Millipore, Billerica, USA
PCR plate strip caps	Thermo Fisher Scientific Inc., Rockford, USA
PCR plates, deep well cycler iQ	Biorad, CA, USA
Pipette tips (1250 µl, 0.1-10 µl and 2-200 µl)	Sarstedt, Nümbrecht, Germany
TruCount tubes	BD Biosciences GmbH, Plymouth, USA
Nunc™ MicroWell™ 96-Well Microplates	Thermo Fisher Scientific Inc., Rockford, USA

3.1.3 Equipment

Table 3-3: Equipment

Equipment	Manufacturer
ABX Micros60	Axonlab AG, Baden, Germany
BD FACSCanto II Flow cytometry	BD Biosciences GmbH, Plymouth, USA
Block Heating System For Micro tubes	Grant, Essex, England
Clean bench Herasafe KS	Thermo Fisher Scientific, Waltham, USA
Counting chamber „Neubauer“	Brand, Wertheim, Germany
Elispot reader + fluorescent lamp	AID GmbH, Straßberg, Germany
FACSDiva™ Flow Cytometry & Cell sorter	BD Biosciences GmbH, Plymouth, USA
Grant water bath GD100	Grand, Shepreth, UK
Heidolph Titramax x10000	neo-Lab, Heidelberg, Germany
Heraeus Fresco 17 centrifuge	Thermo Fisher Scientific Inc., Rockford, USA
Incubator Heracell 240i	Thermo Fisher Scientific, Waltham, USA
Labor centrifuge 5415D	Eppendorf AG, Hamburg, Deutschland
Laboratory shaker	Neolab, Heidelberg, Germany
MACS LS Columns	Miltenyi Biotec GmbH, Gladbach, Germany

MACS MultiStand	Miltenyi Biotec GmbH, Gladbach, Germany
Megafuge 1.0	Heraeus Instruments, Hanau, Germany
MidiMACS Separator LS	Miltenyi Biotec GmbH, Gladbach, Germany
Multichannel Pipette	Biohit, Rosbach, Germany
Multifuge X3R	Thermo Fisher Scientific, Waltham, USA
Multistep pipette	Brand, Wertheim, Deutschland
Optical microscope	Olympus, Hamburg, Germany
pH-meter Schott Instruments	BDL Czech Republic, Trutnov, Czechia
Pipetboy	IBS Integra Biosciences, Fernwald, Germany
Pipettes (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl)	Eppendorf AG, Hamburg, Germany
Refrigerators and freezer (4°C, -20°C, -80°C)	Liebherr Heraeus Instruments, Hanau, Germany
Shaker Titramax 1000r	Heidolph, Schwabach, Germany
Shandon CytoSpin III Cytocentrifuge	GMI, Minnesota, USA
SpectraMax190 microplate reader	molecular devices, Sunnyvale, CA, USA
T100 Thermo Cycler	Biorad, CA, USA
Zeiss LSM 710 confocal microscope	Carl Zeiss, Jena Germany

3.1.4 Buffer and solutions

Developer

Solution A

- 10 ml CH_3COOH 0.2 M
- 14.1 ml $NaOOC\text{-}CH_3 * 3\ H_2O$ 0.2M
- 20 ml dest. water

Solution B

- 1 tablet AEC in 2 ml DMSO

add 1 ml AEC-DMF stepwise to the solution A and after filtration add 12 µl H_2O_2 (30%) to the solution

Coating Buffer: in 500 ml dest. water pH 9.6

- 1.46 g $NaHCO_3$
- 4.5 g NaCl
- 0.8 g Na_2CO_3

Loading Buffer electrophoresis: in 11 ml dest. water

- 1.8 ml Loading dye
- 1 ml Glycerin
- 5 µl GelRed

Phosphatase storage buffer

- 25 mM Tris-HCl (pH 7.4)
- 2 mM EDTA
- 10 mM β-Mercaptoethanol

Removal buffer: in PBS

- 1% BSA
- 0.05% Tween

20xTBS: in 500ml dest. water pH 7.4

- 121 g Tris
- 90 g NaCl

10x TBE electrophoresis buffer: in 1 l dest. water

- 121.1 g Tris
- 61.8 g Boric acid
- 7.4 g EDTA

3.1.5 Antibodies

Table 3-4: Antibodies. If not otherwise indicated, all antibodies listed below are from mouse against human.

antibody	conjugate	clone	company	Application field	dilution
CD3	PacB	UCHT1	BD	FC, cell sorting	1:50
CD14	PacB	M5E2	BD	FC, cell sorting	1:50
CD19	PE Cy7, APC-H7	SJ25C1	BD	FC, cell sorting	1:15, 1:12
CD20	PerCP-Cy5.5, PacO	H1 (FB1), HI47	BD, Invitrogen	(intracellular) FC, cell sorting	1:12, 1:50
CD21	PE	B-ly4		FC	1:25
CD22	PE	S-HCL-1	BD	FC	1:20
CD27	APC	HIT2	BD	FC	1:30
CD38	PE	HIT2	BD	FC, cell sorting	1:20
CD80	FITC	B7-1	BD	FC	1:20

CD86	FITC	2331 (FUN-1)	BD	FC	1:50
CD95	PE	DX2	BD	FC	1:50
CD138	PE	B-B4	Miltenyi	FC	1:25
donkey anti-goat IgG	FITC		Jackson Research	CM	1:100
donkey anti-mouse IgG	RRX		Jackson Research	CM	1:200
donkey anti-rabbit IgG	RRX		Jackson Research	CM	1:200
Epratuzumab (anti-CD22)	Bio		UCB	CM	1:10
$F(ab')_2$ goat anti-IgM/ IgG			Jackson Research	CM, stimulation	1:25, 1:100
goat anti-IgG (γ-chain specific)	unlabeled		Sigma-Aldrich	ELISA, ELISPOT	1:200
IgD	PE-Cy7	IA6-2	BD	FC	1:50
IgA	FITC	M24A	Chemicon	FC	1:50
IgG	PE-Cy7	G18-145	BD	FC	1:50
IgM	PerCp5.5	G20-127	BD	FC	1:50
IgG	Bio		Sigma-Aldrich	ELISPOT	1:200
IgG	AP		Sigma-Aldrich	ELISA	1:3000
Ki67	PE-Cy7	B56	BD	(intracellular) FC	1:50
p-Akt (S473)	PE	M89-61	BD	(intracellular) FC	1:16
p-CD22(Y822)	PE	12A/CD22	BD	(intracellular) FC	1:6
p-Syk(Y352)	PE	17A/P-ZAP70	BD	(intracellular) FC	1:6
p-Syk (Y348)	PE	II20-722	BD	(intracellular) FC	1:6
p-PLC-γ2 (Y759)	FITC	K86-689.37	BD	(intracellular) FC	1:6
rabbit anti-IgM	unlabeled		DAKO	CM	1:200
SHP-1	unlabeled	52/PTP1C	BD	CM	1:200
Streptavidin	Alexa633		Jackson Research	CM	1:200
Streptavidin	HRP		Sigma-Aldrich	ELISPOT	1:3000
Syk	FITC	4D10	BD	(intracellular) FC, CM, cell sorting	1:100

FC= flow cytometry; **CM**= confocal Microscopy; **BD**= Becton Dickinson Biosciences GmbH, Plymouth, USA

3.1.6 Primers

Table 3-5: Primers used for single cell PCR

	Sequence
external PCR	
V_H1 LC	5' CC ATG GAC TGG ACC TGG A 3'
V_H2 LC	5' ATG GAC ACA CTT TGC T(AC)C AC 3'
V_H3 LC	5' CC ATG GAG TTT GGG CTG AGC 3'
V_H4 LC	5' ATG AAA CAC CTG TGG TTC TT 3'
V_H5 LC	5' ATG GGG TCA ACC GCC ATC CT 3'
V_H6 LC	5' ATG TCT GTC TCC TTC CTC AT 3'
for IgA:IgV_H-cα	5' GGA AGA AGC CCT GGA CCA GGC 3'
for IgG: Ecγ	5' AC GCC GCT GGT CAG GGC GC 3'
for IgM: Ecμ	5' TCA GGA CTG ATG GGA AGC CC 3'
interne PCR	
V_H1 FM	5' CAG GTG CAG CTG GTG CAG TCT GG 3'
V_H2 FM	5' CAG GTC ACC TTG AAG GAG TCT GG 3'
V_H3 FM	5' GAG GTG CAG CTG GTG GAG TCT GG3'
V_H4 FM	5' CAG GTG CAG CTG CAG GAG TCG GG3'
V_H5 FM	5' GAG GTG CAG CTG GTG CAG TCT GG3'
V_H6 FM	5' CAG GTA CAG CTG CAG CAG TCA GG3'
IgA	5' ACC AGG CAG GCG ATG ACC AC 3'

IgG	5' AAG TAG TCC TTG ACC AGG CAG C 3'
IgM	5' AGG AGA CGA GGG GGA AAA GGG TTG 3'

OD= optical density; **MW**= molecular weight

3.1.7 Kits

Table 3-6: Kits

Kit	Manufacturer
Tyrosine and serine/threonine Phosphatase assay	Promega, Madison, WI, USA
AnnexinV, PI staining kit	Miltenyi Biotec GmbH, Gladbach, Germany Stem
Rosette Sep(R) depletion kit for CD3 and CD36	Roche Diagnostics GmbH, Mannheim, Germany
Titan One tube RT-PCR System	Sanquin Reagents, Amsterdam, Netherlands
PeliKine Compact™ human soluble CD27 ELISA	Qiagen, Hilden, Germany
QIAquick gel extraction	Roche Diagnostics GmbH
AmpliTaq DNA Polymerase kit components	Generic Assay, Berlin, Germany
Hep-2 immunofluorescence assay	Miltenyi Biotec GmbH, Gladbach, Germany
B cell isolation Kit II (human)	Miltenyi Biotec GmbH, Gladbach, Germany

3.1.8 Software

Table 3-7: Software

Software	Manufacturer
FlowJoTM 7.6.5 software	Tree Star, Ashland, CR, USA Miltenyi
Zen 2011 light edition software	Carl Zeiss, Jena Germany
GraphPad Prism4 software	GraphPad, San Diego, CA
Chromas 2.33 sequence viewer	Chromas Technelysim, Helensvale, Australia; link: http://joinsolver.niaid.nih.gov/
Joinsolver® software	Tree Star, Ashland, CR, USA Miltenyi

3.2 Patients and Methods

3.2.1 Patients

The study was approved by the local ethics committee of the Charité University Hospital Berlin and written consent was obtained by all patients and controls. EDTA or heparin anti-coagulated peripheral blood was taken from 50 healthy donors (HD) (40 females/10 males) with a mean age of 35 years (range 25-58 years) and 82 patients suffering from SLE (76 females/6 males) with a mean age of 43 (range 22-79 years). The disease activity of the latter was assessed by the SLEDAI and was 7.7 (Range 1-32) which was calculated based on 25 different clinical and medical domains. All patients received immunosuppressive drugs; methotrexate, prednisone, azathioprine and mycophenolate mofetil. In addition, 15 rheumatoid arthritis (RA) patients (13 women, 2 men) mean age of 49, range 27-71 years) with a mean disease activity score (DAS 28) of 3.2 (range 1.8-4.9) and 20 female primary Sjögren's syndrome (pSS) patients (all anti-Ro/La Ab positive; mean age of 56, range 31-76 years) served as controls. For each blood sample the

differential cell count (measured by Micros60 **Table 3-3**) and the total B cell count (**3.2.4**) were determined.

3.2.2 Cell isolation

3.2.2.1 Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated by Ficoll density-gradient centrifugation. According to their specific density, lymphocytes and monocytes will be concentrated at the interphase between the plasma phase and the Ficoll layer. Erythrocytes and granulocytes will form a pellet because of their higher density. EDTA anti-coagulated whole blood or buffy coat was diluted 1:2 with PBS. 30 ml blood-PBS suspension was layered on 15 ml lymphocyte separation medium and centrifuged for 20 min at RT at 840xg w/o brake. The PBMC concentrated in the interphase, were collected in a fresh 50 ml tube and washed two times with 50 ml cold PBS/0.5% BSA using a centrifuge at 400xg for 10 min. After cell counting with a counting chamber (Neubauer), cells were resuspended either in pre-warmed RPMI for stimulation assays, in RPMI/10% FCS for *in vitro* cultures or in cold PBS/0.5% BSA for FACS analysis and B cell purification. Trypan blue was used to exclude dead cell during the counting process.

3.2.2.2 B cell purification

B cells isolated from PBMC were purified using the human B cell isolation Kit II according to the manufacture's protocol (**Table 3-6**).

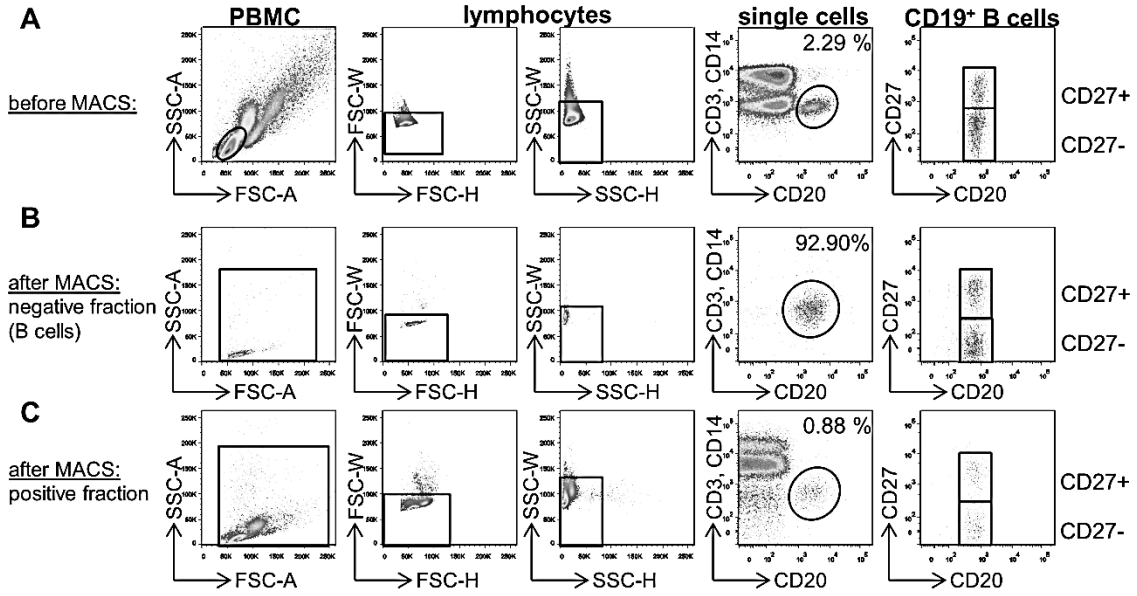

Figure 3-1: Gating strategy and flow cytometric analysis of PBMC before and after B cell purification. A, PBMC were isolated from whole blood. After duplet exclusion lymphocytes were gated regarding their expression of CD3, CD14 and CD20, to identify the frequency of total CD20+ B cells (2.29%). Non-B cells were labeled with biotin conjugated Ab and anti-biotin magnetic beads. **B,** Magnetic labeled non-B cells bound to the magnetic column (positive fraction) whereas negative target B cell population runs through with a B cell purity of 92.90%. **C,** The positive fraction bound to the magnetic column contained only a small fraction of remaining B cells (0.88%).

The B cell isolation Kit II labels non-B cells with magnetic beads and untouched B cells can be purified. Non-B cells (T cells, NK cells, monocytes, DCs, granulocytes and elytroid cells) were labeled with biotin conjugated Ab and afterwards by anti-biotin MicroBeads. These magnetic labeled cells bound to a magnetic MACS column and the negative target population (B cells) will remain untouched and appeared in the efflux fraction. Afterwards, the cells were washed (8 min at 250xg) with PBS/0.5% BSA/2 mM EDTA and resuspended in PBS. B cells were counted and an aliquot was used for the purity check by FACS analyses (**3.2.6**). Trypan blue was used to exclude dead cell during the counting process. A total B cell purity over 90% (**Figure 3-1**) was revealed as pure and was used for further B cell specific analysis. Cells were kept on ice for further assays.

3.2.3 Flow Cytometry (FC)

Flow cytometry, a fluidic-and laser-based method, was used to perform multi-parameter analysis on single cells. Besides the cell size and granularity, the expression of various surface and intracellular markers were determined by labeling cells with fluorochrome-conjugated Ab specific for the marker of interest. Specific conjugated monoclonal Ab (**Table 3-4**) were used in a final staining volume of 50 µl. The staining time was depending on the Ab used. As a quality control, CST beads and rainbow beads according to the

manufacture's instructions were measured before each FC analysis to ensure stable MFI values for the expression of surface markers and intracellular kinases over time.

3.2.4 Determination of the total B cell count (TruCount)

For the determination of the absolute B cell number, a specialized TruCount FACS tube was used which contains a pellet with a defined number of fluorescent beads. 20 µl TruCount Ab Mix (**Table 3-8**) and 50 µl anti-coagulated whole blood was pipetted in the TruCount tube and incubated at RT for 15 min. Afterwards, 450 µl TruCount Lysis solution was added incubated for additional 15 min at RT and cells were analyzed by FC using Canto II flow cytometer.

Table 3-8: TruCount Ab Mix

antibody	conjugate	clone	company	Volume [µl]
CD14	PacB	MT1	BD	1
CD19	PE-Cy7	SJ25C1	BD	2
CD20	FITC	H147	BD	2
CD45	PerCp	2D1	BD	3,5
dest. water				11,5

3.2.5 Intracellular staining

For the analysis of different receptors and intracellular kinases regarding their phosphorylation and expression profile, 500 µl EDTA anti-coagulated whole blood or $1x10^6$ stimulated PBMC were immediately fixed with pre-warmed 1x Lyse/Fix Buffer for 10 min at 37°C and subsequently washed with 4 ml ice cold PBS for 8 min at 600xg. Afterwards, cells were permeabilized with Perm Buffer II or III over night at -20°C or 30 min on ice. After washing two times with 4 ml cold PBS/1% FCS for 8 min at 600xg cells were resuspended in 50 µl PBS/1% FCS and stained for 1h at RT (CD3-PacB, CD14-PacB, CD19-APH-H7, CD20-PacO, CD27-APC and CD38-PE or CD22-PE, p-CD22-PE, p-Syk-PE, Syk-FITC, CD80-FITC, CD86-FITC, CD95-PE, Ki67-PE-Cy7, IgD-PE-Cy7 or CD21-PE, respectively), subsequently washed with 2 ml PBS/1% FCS and analyzed by FC using Canto II flow cytometer. Data were analyzed using FlowJo™ 7.6.5. software (**Figure 3-2A**).

3.2.6 Surface staining/purity check

Cells were resuspended in 50 µl PBS/0.5% BSA and stained for 15 min at 4°C (purity check: CD3-PacB, CD14-PacB, CD19-APC-H7, CD20-PacO and CD27-APC). After washing 5 min at 350xg once with PBS/0.5 % BSA, 1µl DAPI was added and cells were analyzed by FC using Canto II flow cytometer (**Figure 3-2B**).

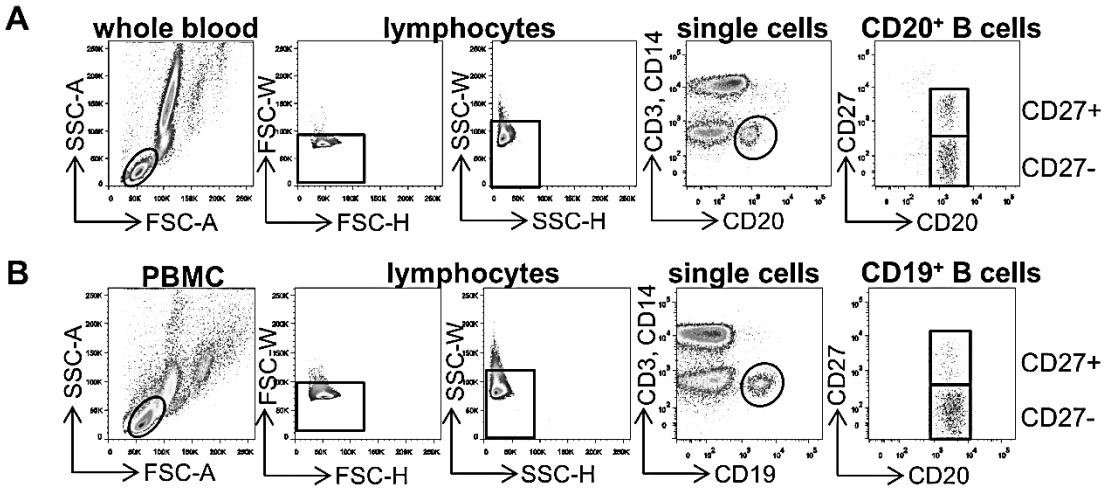

Figure 3-2: Gating strategy and flow cytometric analysis. A, Whole blood was fixed, lysed, permeabilized and duplets were excluded. Lymphocytes were gated on their expression of CD3, CD14 and CD20, to identify the expression of different markers or intracellular kinases within CD20+CD27+ and CD20+CD27- B cells. **B,** After Ficoll, single lymphocytes were gated based on their expression of CD3, CD14 and CD19 to identify the expression of different markers in total CD20+CD27+ and CD20+CD27- B cells.

3.2.7 Flow cytometric analysis of apoptosis by AnnexinV

For the analysis of viable, apoptotic and necrotic B cells after BCR stimulation, an AnnexinV and propidium iodide (PI) staining was performed. AnnexinV binds to phosphatidylserine (PS) which is located on the cytoplasmic surface of the cell membrane in viable cells. In cells undergoing apoptosis PS translocate, to the outer leaflet of the membrane, and accessible for AnnexinV binding. Apoptotic but not necrotic cells are PI negative, since only necrotic cells with a permeable cell membrane leak PI. Therefore, viable cells (AnnexinV and PI double negative) can be distinguished from apoptotic (AnnexinV positive, PI negative) and necrotic (AnnexinV and PI double positive) cells. $1x10^6$ PBMC from SLE patients and HD were pre-incubated for 1h at 37°C in RPMI and subsequently stimulated with 12 µg/ml anti-IgM/IgG for 5 or 60 min at 37°C. As an AnnexinV negative control unstimulated cells were used. For a positive AnnexinV control cells were stimulated for 5 min with 3% H_2O_2. PBMC were immediately put on ice and washed with AnnexinV binding buffer. Afterwards cells were stained for 30 min on ice in the dark (CD3-PacB, CD14-PacB, CD19-PE-Cy7, CD20-PacO, CD27-APC and

AnnexinV). Stained cells were washed again with AnnexinV binding buffer and prior to analysis PI was added to the cells. The frequency of AnnexinV/PI double negative cells represented the proportion of viable cells.

3.2.8 Intracellular phosphorylation kinetics

PBMC were isolated from SLE patients and HD and resuspended in RPMI ($10x10^6$ PBMCs/ml). $1x10^6$ cells were pre-incubated in a water bath for 1h at 37°C and subsequently stimulated with 12 µg/ml anti-IgM/IgG for different time points. PBMCs were immediately fixed after stimulation with 1 ml pre-warmed 1x Lyse/Fix Buffer and permeabilized with 200 µl Perm buffer II or III (**3.2.5**). Cells were afterwards stained at RT for 1 h (CD3-PacB, CD14-PacB, CD19-PE-Cy7, CD20-PerCp5.5, CD27-APC, Syk-FITC or p-PLC-γ2(Y759)-FITC and p-Syk(Y352)-PE or p-Syk(Y348)-PE, p-CD22(Y822)-PE, p-Akt(S473)-PE, respectively).

3.2.8.1 After in vitro culture (resting)

$1x10^6$ PBMC from SLE patients and HD were incubated for 24 h and 48 h at 37°C/5% CO_2 in RPMI/10% FCS medium and subsequently stimulated at 37 °C with 12 µg/ml anti-IgM/IgG for 5 min. PBMC were immediately fixed and permeabilized after stimulation (**3.2.5**) and stained 1h at RT (CD3-PacB, CD14-PacB, CD20-PerCp5.5, CD27-APC, p-Syk(Y352)-PE and Syk-FITC).

3.2.9 Confocal microscopy

3.2.9.1 Whole blood analysis

For analysis of the cellular distribution of intracellular Syk, 10 ml EDTA anti-coagulated whole blood from patients with SLE was fixed, lysed and permeabilized in 50 ml Lyse/Fix and 5 ml Perm II Buffer as described before (**3.2.5**). Cells were stained at RT for 1 h (CD3-PacB, CD20-PerCp5.5, CD27-APC and Syk-FITC). Three CD20+ B cell populations were sorted: CD27-Syk+, CD27-Syk++ and CD27+ by using the FACSAria™ cell sorter. $0.15x10^5$ sorted B cells were incubated with the primary Ab rabbit anti-human IgM at 5 µg/ml for 40 min at RT. Subsequently, cells were washed once with 3 ml PBS/1% FCS and stained with the secondary Ab donkey anti-rabbit IgG Rhodamine Red X (RRX) for 40 min at RT. Cells were centrifuged at 250xg for 1 min and covered with Vectashield

Hard set mounting medium with DAPI. Afterwards, the cells were measured using Zeiss LSM 710 confocal microscopy (magnification: 630x). For quantitative determination, the subcellular distribution by single cells was analyzed with Zen 2011 light edition software.

3.2.9.2 BCR stimulation analysis

B cells were enriched from whole blood before sorting using RosetteSep® Human CD3 and CD36 depletion Cocktail according to the manufacturer's protocol. Briefly, 20 µl anti-CD3 and 20 µl anti-CD36 tetrameric Ab complexes were added to 10 ml EDTA whole blood and incubated at RT for 20 min. CD3+ T cells and CD36+ monocytes were cross-linked to red blood cells by the tetrameric Ab complexes recognizing simultaneously CD3 or CD36 and the red blood cell specific marker glycophorin A. After separation by Ficoll density-gradient centrifugation the labeled CD3+ and CD36+ cells together with the red blood cells formed a pellet because of their higher density. B cell enriched lymphocytes will be concentrated at the interphase between plasma and Ficoll. $0.5x10^6$ B cell enriched PBMC were incubated at 37°C for 1 h and subsequently stimulated with 12 µg/ml goat anti-human IgM/IgG for 5 min. Afterwards cells were fixed and permeabilized as recorded previously (**3.2.5**). For analyzing the capping and recruitment of SHP-1 to CD22 and CD22 to the BCR, cells were stained at RT for 40 min with epratuzumab-biotin (bio, UCB), goat anti-human IgM/IgG (unstimulated control only) and mouse anti-human SHP-1. Cells were washed once with 3 ml PBS/1% FCS and stained at RT for 40 min with the secondary Ab donkey anti-goat FITC, Streptavidin-Alexa 633 and donkey anti-mouse RRX. Fixed, permeabilized and stained cells were centrifuged at 250 g for 1 min on a slide and covered with Vectashield Hard set mounting medium with DAPI. Afterwards, the cells were measured using Zeiss LSM 710 confocal microscopy (original magnification: 630x). For quantitative determination, the co-localization coefficient by single cells (CD22+, IgM+/IgG+ B cells) were analyzed with Zen 2011 light edition software.

3.2.10 Calcium flux assessment

B cells were purified by using human B cell isolation Kit II (**3.2.2.2**). $0.5x10^6$ B cells were washed in RPMI/10% FCS and loaded with Indo-1 acetoxymethyl (AM) dye at 0.2 µM at

37°C for 30 min. B cells were washed with PBS/0.5% BSA and stored on ice. Measurements were performed by using a LSR II flow cytometer. Before stimulation B cells were pre-incubated at 37°C for 5 min and afterwards the baseline was recorded for 40 sec, then 12 µg/ml anti-IgM/IgG was added and changes in the intracellular calcium (Ca^{2+}) concentration were recorded for 6 min. The intracellular Ca^{2+} concentration was quantified by calculating the ratio of 405 nm/ 475 nm Indo-1 AM emission (bound/unbound indo).

3.2.11 Tyrosine and serine/threonine phosphatase activity assay

B cells isolated from HD and SLE patients were purified using human B cell isolation Kit II as indicated above (**3.2.2.2**). $1x10^6$ purified B cells were lysed in 100 µl Pierce IP Lysis buffer supplemented with 1% protease inhibitor cocktail on ice for 30 min and centrifuged afterwards 15 min 17.000xg at 4°C. The lysate was diluted 1:4 with phosphatase storage buffer (177). Supernatants were analyzed regarding their phosphatase activity by using the phosphatase assay system kit according to the manufacture's instruction (tyrosine and serine/threonine phosphatase assay, **Table 3-6**). Endogenous phosphate was removed from the lysate by Sephadex G-25 spin columns supplied with the kit. Specialized phosphorylated peptides for tyrosine and serine/threonine phosphatases were used as substrate (Tyrosine-1: END(pY)INASL, Tyrosine-2: DADE(pY)LIPQQG, Serine/Threonine: RRA(pT)VA). Each lysate was incubated with or without 100 µM substrate for 15 min at 37°C and free phosphate which was released during the reaction was detected by a molybdate solution at 600 nm by using a SpectraMax190 microplate reader. The background (lysate without phosphopeptide) was subtracted. A phosphate standard curve was generated and used as reference for the B cell lysates. As control, phosphatase inhibitors were used (10 mM sodium fluoride or sodium orthovanadate).

3.2.11.1 Preparation of sodium orthovanadate

A 100 mM sodium orthovanadate solution at pH of 10 was prepared and boiled until the solution was colorless. Afterwards the solution was cooled down on ice and the pH was calibrated again (pH 10). If the solution turns yellow/orange again after cooling, boiling and cooling process have been repeated as well as pH adjustment until the solution appeared to be colorless during the cooling procedure with a final pH of 10.

3.2.11.2 Tyrosine phosphatase inhibition for intracellular phosphorylation analysis

$1x10^6$ isolated PBMC from SLE patients and HD were pre-incubated for 1h at 37°C in RPMI, pre-treated with 10 mM of sodium orthovanadate for 5 min at 37°C and subsequently stimulated with 12 µg/ml anti-IgM/IgG for 5 min. PBMC were immediately fixed and permeabilized (**3.2.5**) and stained 1h at RT (CD3-PacB, CD14-PacB, CD20-PerCp5.5, CD27-APC, p-Syk(Y352)-PE and Syk-FITC).

3.2.12 Tetanus (TT) vaccination

During a TT vaccination study 6 HD (3 female and 3 male; average age of 36 with an age range of 26-55) underwent secondary immunization against TT/ diphtheria toxoid (20 immunization units TT and 2 immunization units diphtheria toxoid). Whole blood was drawn and analyzed at day 0, 6, 7 and 14 after vaccination with the tetanus/diphtheria toxoid to assess the frequency of CD27-Syk++ and CD19+CD27++ B cells. Therefore whole blood was fixed and permeabilized as described previously (**3.2.5**) and stained 1h at RT (CD3-PacB, CD14-PacB, CD19-PE-Cy7, CD20-PacO, CD27-APC and Syk-FITC).

3.2.13 ATP-binding cassette (ABC)-B1 transporter activity

For the analysis of the ABC-B1 transporter activity a MitoTrackerOrange (MTO) staining was performed. MTO is an orange-fluorescent dye that stains mitochondria and accumulates in live cells. The ABC-B1 (P-glycoprotein) transporter is an ATP dependent drug pump and decreases the accumulation of different drugs including MitoTrackerOrange in the cell. If cells express an active ABC-B1 transporter, like naïve CD27- B cells, MTO will not accumulate in these cells. Therefore, those cells would be negative for MTO. In contrast, memory (CD27+) or transitional (CD27-CD24++CD38++) B cells show an inactive ABC-B1 transporter. The MTO dye accumulates in these cells (MTO positive). 100 µL EDTA anti-coagulated whole blood was incubated with 1 mM MTO CMTMRos for 30 min at 37°C and subsequently fixed, lysed and permeabilized with Perm Buffer II 30 min on ice as delineated before (**3.2.5**). Afterwards cells were stained at RT for 1 h (CD3-PacB, CD20-PerCp5.5, CD27-APC, CD38-PE and Syk-FITC). FC

analysis was performed after one washing step using Canto II flow cytometer. Data were then analyzed using FlowJo™ 7.6.5. software.

3.2.14 Real time (RT)-PCR of immunoglobulin mRNA from single cells

3.2.14.1 Single cell sort and cDNA synthesis

Fixed and permeabilized PBMC according to the manufacturer's protocol from BD (3 patient samples SLE#1-SLE#3) were incubated for 1 h at RT using the following anti-human Ab: CD3-PB, CD14-PB, CD19-PECy7, CD27-APC and Syk-FITC. For SLE#4 isolated PBMC were incubated for 15 min at 4°C using the following anti-human Ab: CD3-PacB, CD14-PacB, CD19-PECy7, CD27-APC and CD38-PE. For each of four SLE patients, single CD3-CD14-CD19+CD27-Syk+ and CD3-CD14-CD19+CD27-Syk++ cells (SLE#1-SLE#3) or CD3-CD14-CD19+CD27-CD38+ and CD3-CD14-CD19+CD27-CD38- cells (SLE#4), respectively, were sorted into a 96-well plate (round bottom) using a FACSAria cell sorter (BD). Each well was pre-loaded with 30 µl reaction mix containing 8.3 mM DTT, 0.8 nM Spermidin, 0.5 µg BSA, 20 U RNasin Plus RNase inhibitor and 1.7% TritonX. After the FACS sort 20 µl of a second mix containing the Titan One Tube kit's RT-PCR buffer and AMV reverse transcriptase (**Table 3-6**), prepared according to the manufacturer's instruction, supplemented with the p(dT)15 Primer and 0.5 mM dNTPs was added and cDNA was generated at 50°C for 60 min.

3.2.14.2 Nested PCR and sequence analysis

To amplify the cDNA of rearranged $V_HDJ_HC\mu$ transcripts a nested PCR protocol was performed. For the external and internal PCR the final reaction mix consisted of the AmpliTaq DNA Polymerase kit components prepared according to the manufacturer's instructions (**Table 3-6**) and supplemented with 0.2 mM dNTPs and 0.06 µM of each of the external and internal primers, respectively. Specific oligonucleotide sequences for the external and internal PCR as shown in **Table 3-5** a forward primer set for V_H1-V_H6 regions as well as a reverse isotype-specific IgA, IgG and IgM primer set was used. 5 µl of the generated cDNA were used as a template for the external PCR. Thermo cycler conditions for the external PCR were as followed: 95°C for 2 min, 50 cycles of 95°C for 1 min, 56°C annealing temperature for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min and a ramping of 2.5°C/s. 5 µl of the external PCR products were used as template

for the internal PCR. The internal PCR was performed in an identical manner, except for the annealing temperature, which was set to 58°C. PCR products were separated by agarose gel electrophoresis, visualized with gelred by exposure to UV light and purified by using a QIAquick gel extraction kit according to the manufacturer's instruction (**Table 3-6**). PCR products were sequenced at Eurofins MWG Operon. The sequences were analyzed using the Chromas 2.33 sequence viewer and the Joinsolver® software (**Table 3-7**).

3.2.15 Plasma cell differentiation *in vitro*

PBMC were isolated and stained at 4°C for 15 min against human CD3-PacB, CD14-PacB, CD19-PE-Cy7, CD27-APC and CD38-PE. Three CD3-CD14-CD19+ B cell populations were sorted: CD27+CD38+/-, CD27-CD38+ and CD27-CD38-, respectively using a BD FACSAria™ cell sorter. Sort-purified B cell populations were seeded with $0.5x10^5$ cells per well (96-well round bottom) and cultured for 5 days at 37°C/5% CO_2 in RPMI/10% FCS in the presence of IL-2 (40 ng/ml), IL-10 (40 ng/ml), anti-human IgM/IgG (2 µg/ml) and CpG oligodeoxynucleotides (2.5 µg/ml, ODN 2006). The supernatants were collected and subsequently frozen at -70°C until further analyses. The cells were washed once with 2 ml PBS/0.5% BSA and stained for 15 min at 4°C (CD3-PacB, CD14-PacB, CD20-PacO, CD27-APC and CD138-PE) for FC analysis or cultured for further ELISPOT experiments.

3.2.16 Detection of immunoglobulin secretion

$0.5x10^5$ sorted B cells according to their expression of CD27 and CD38 were seeded per well and cultured with IL-2 (40 ng/ml), IL-10 (40 ng/ml), anti-human IgG/IgM (2 µg/ml) and CpG oligodeoxynucleotides (2.5 µg/ml, ODN 2006) for 5 days at 37°C/ 5% CO_2. Supernatants were collected and levels of total IgG were measured by an enzyme-linked immunoabsorbent assay (ELISA) or the frequency of spontaneous IgG-producing cells was determined using ELISPOT.

3.2.17 ELISPOT

An ELISPOT plate was prepared using 15 µl of 35% ethanol per well. Afterwards the plate was washed 3 times with 100 µl PBS and coated with 50 µl anti-human IgG (5µg). The

plate was incubated over night at 4°C and washed afterwards 3 times with 100 µl PBS. Membranes were blocked with 100 µl TBS/2% BSA and 100µl 1:1 RPMI/10% FCS for 1 h at 37°C and washed once. Cultured cells were washed two times with 200 µl cold RPMI (8 min 350xg) and a cell serial dilution (1:3, 1:9, 1:27, 1:81, 1:243, 1:729 and 1:2187) with a total volume of 150 µl was plated. As positive control, 10 µl serum from a HD was used. After 3 ½ h incubation at 37°C/ 5% CO_2, plate was washed 6 times with cell removal buffer. For the detection, 50 µl anti-IgG-bio was added and incubated for 1h at RT. After washing with PBS 50 µl Streptavidin-HRP (30 min, RT) and thereafter 100 µl of the staining solution were added. The plates were incubation 5 min at RT (dark), washed with dest. water and dried over night. Dots were analyzed with an AID UV ELISPOT reader.

3.2.18 ELISA

96 well plates (NUNC, Germany) were coated (2 h, 37°C) with 50 µl anti-human IgG (5 µg) in coating buffer. After removing the coating buffer plates were blocked with 200 µl TBS/2% BSA (1h, RT). Afterwards, the plates were washed and 50 µl culture supernatant or standard was pipetted and incubated on the shaker (2 h, RT, 300 rpm). Plates were washed 3 times with 200µl TBS/0.05% Tween and incubated with 100 µl anti-IgG-AP (2 h, RT). Washing steps were repeated and finally the plates were incubated for 30 min at RT with 100 µl substrate (2 pNPP tablets dissolved in 10 ml Ethanolamine). The optical density (OD) was measured (405 nm) using a SpectraMax190 microplate reader.

3.2.19 Detection of auto-IgG-Ab by indirect Hep-2 immunofluorescence assay

The production of auto-Ab against nuclear and cytoplasmic antigens of sorted and cultured B cells (**3.2.16**) was analyzed by using indirect Hep-2 immunofluorescence assays (**Table 3-6**). 25 µl of supernatant (5 days cell culture), positive and negative control (provided with the kit) were incubated for 30 min on immobilized Hep-2 cells. After three washing steps with PBS, the slides were incubated for 30 min with FITC labeled anti-human IgG Ab (provided with the kit). The cells were afterwards covered with Vectashield Hard Set mounting medium with DAPI. Bound auto-Ab were detected using Zeiss LSM 710 confocal microscope (x630 magnification) and processed by Zen2011 light edition software.

3.2.20 Whole blood cell culture

100 µl whole blood (Heparin) per well (96-well round bottom) were cultured at 37°C/5% CO_2 in the presence of BAFF (200 ng/ml), IFN-γ (200 ng/ml), TNF-α (200 ng/ml), IL-6 (200 ng/ml), IL-21 (200 ng/ml), LPS from Escherichia coli 055:B5 (2.5 µg/ml) or CpG oligodeoxynucleotides (2.5 µg/ml, ODN 2006) for 24 h. Afterwards the blood was fixed and permeabilized as described previously (**3.2.5**). Cells were stained at RT for 1 h (CD3-PacB, CD14-PacB, CD19-PE-Cy7, CD20-PerCp5.5, CD27-APC and Syk-FITC) and analyzed by flow cytometry (FC) using a BD Canto II flow cytometer. Data were subsequently analyzed by using FlowJo™ 7.6.5. software.

3.2.21 sCD27 ELISA

A 96 well plate provided by the PeliKine Compact TM human soluble CD27 ELISA kit (**Table 3-6**) was coated with 100 µl anti-sCD27 in coating buffer at 4°C overnight. After removing the coating buffer plates were blocked with 200 µl TBS/2% BSA 1 h at RT. Afterwards, plates were washed twice with 100 µl 1x TBS and 100 µl of culture supernatant (1:1), serum (1:5) or standard (serial dilution: range 100-3.125 U/ml) were diluted in TBS/0.5% BSA, pipetted on the plate and incubated for 2 ½ h at RT on the shaker (300 rpm), washed 3 times with 200 µl TBS/0.05% Tween and incubated with 100 µl anti-sCD27-Bio (1½ h, RT). Washing steps were repeated and the plates were incubated for 1 h at RT with 100 µl Streptavidin-HRP. After washing (TBS/0.05% Tween) 100 µl 1-Step TMB-ELISA solution was added (15 min, RT). Reaction was stopped with 100 µl 1.8 M H_2SO_4 and the OD was measured (450 nm) using a SpectraMax190 microplate reader.

3.2.22 Statistical analysis

The statistical analysis was performed with GraphPad Prism4 software (GraphPad, San Diego, CA). Non-Gaussian distributed sets of paired data were analyzed using Wilcoxon signed-rank-test; other non-parametric data were compared using the Mann-Whitney test, and Fisher's exact test was used for binary data analysis (contingency table). P values less than 0.05 were considered statistically significant. The data are shown as mean±SD if not otherwise indicated.

4. Results

4.1 Dysbalanced BCR signaling in SLE B cells

4.1.1 Increased expression and activation of the inhibitory BCR co-receptor CD22 and disturbed CD19/CD22 balance in CD27- SLE B cells

The inhibitory co-receptor CD22 which is exclusively expressed on B cells plays a pivotal role during B cell differentiation and development (83). The development dependent CD22 expression regulates the signaling threshold and plays a crucial role in controlling peripheral tolerance (79, 83, 178). To assess the expression and activation of the inhibitory co-receptor CD22 in CD27+ and CD27- B cells, whole blood from 30 SLE patients and 22 HD was fixed, permeabilized and stained for CD22 and phosphorylated (p)-CD22 on tyrosine (Y) 822. Comparing CD27+ and CD27- B cells for their CD22 expression showed enhanced expression levels of CD22 on CD27- compared to CD27+ HD and SLE B cells in their mean fluorescence intensity (MFI; **Figure 4-1A**). However, the expression of CD22 between HD and SLE patients revealed a significant increased expression of CD22 on CD27- but not CD27+ SLE B cells (HD: CD27+ 3849±1474, CD27- 7018±2486; SLE: CD27+ 4674±1654, CD27- 8546±2571; mean±SD, *$p<0.05$).

No differences between whole blood CD27+ and CD27- B cells of HD and SLE patients was observed for the phosphorylation of CD22(Y822) which is responsible for the recruitment of the phosphatase SHP-1 (107) (**Figure 4-1B**).

CD19, another BCR co-receptor, also plays an important role in regulating the BCR response. Indeed, disturbances of the balance between CD19 and CD22 (CD19/CD22 loop) has been considered to be involved in autoimmune diseases (115, 116). Therefore, the expression of CD19 and the ratio of CD19 and CD22 in whole blood of SLE patients and HD were analyzed (**Figure 4-1C**). Although SLE and HD B cells express the same levels of CD19, the ratio of CD19 and CD22 showed a significant reduced balance of these two co-receptors on CD27- SLE B cells with a shift towards the inhibitory co-receptor CD22 (**Figure 4-1D**). Here, the ratio of CD19/CD22 in CD27- B cells of HD was 0.10±0.01 and of SLE patients 0.07±0.1 (mean±SD, ***$p<0.001$).

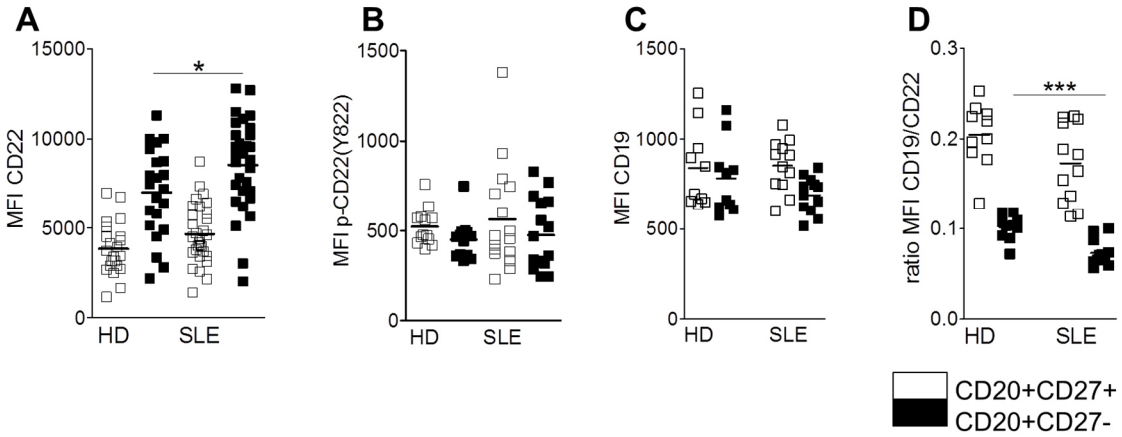

Figure 4-1: CD27- B cells from SLE patients expressed enhanced levels of CD22 and showed simultaneously a disturbed CD19/CD22 balance. Whole blood CD27+ (open square) and CD27- (filled square) B cells obtained from systemic lupus erythematosus (SLE) patients and healthy donors (HD) were analyzed regarding their CD22 (**A,** HD n=22; SLE n=30), p-CD22(Y822) (**B,** HD n=13; SLE n=16) and CD19 (**C,** HD n=10, SLE n=12) expression as well as their ratio of the mean fluorescence intensity (MFI) of CD19 and CD22 (**D,** HD n=10; SLE n=12) revealing an enhanced expression of CD22 and a reduced balance of CD19/CD22 expression in CD27- B cells from SLE patients. Each symbol represents an individual subject; horizontal lines show the mean; ***p>0.001, *p<0.05 by Mann-Whitney U test.

In order to gain insights into potential intrinsic signaling abnormalities in SLE B cells and to analyze to which extent the increased expression of CD22 and disturbed CD19/CD22 balance influences the activation of CD22, the phosphorylation kinetic of CD22(Y822) after BCR stimulation (**Figure 4-2**) as well as the co-localization of SHP-1 and the BCR with CD22 (**Figure 4-3**) were studied in B cells from SLE patients versus HD. Here analyses of the phosphorylation kinetic of CD22 after BCR stimulation showed a significantly increased phosphorylation level in CD27- SLE B cells compared to HD (**Figure 4-2**). After 15 and 30 min of BCR stimulation, CD27- SLE B cells displayed a significantly increased p-CD22 MFI of 913±188 (15 min) and 921±272 (30 min) compared to 633±136 (15 min) and 617±133 (30 min) in CD27- HD B cells (mean±SD, **p<0.01). Additionally, comparing the phosphorylation of CD22 after BCR activation in CD27+ versus CD27- B cells revealed an enhanced activation in CD27+ B cells in HD but not in SLE patients. Indeed, SLE CD27- B cells expressed higher levels of p-CD22 after BCR activation compared to CD27+ SLE B cells (15 min SLE: CD27+ 880±181, CD27- 913±188; HD: CD27+ 857±186 CD27- 633±136, mean±SD; **Figure 4-2B**). This data are consistent with the enhanced expression of CD22 and shifted balance towards CD22 in CD27- SLE B cells (**Figure 4-1**).

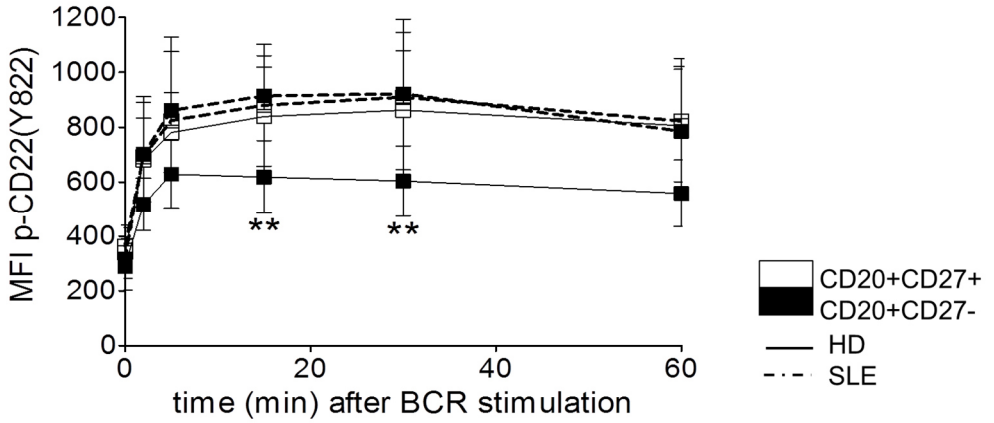

Figure 4-2: Significantly enhanced activation of the inhibitory co-receptor CD22 by CD27- SLE B cells upon 15 and 30 min of BCR stimulation. PBMC from 7 SLE patients and 7 HD were incubated with anti-IgM/IgG for 0, 2, 5, 15, 30 and 60 min. CD27+ (open square) and CD27- (filled square) B cells from SLE patients (dotted) compared to HD (solid) were analyzed regarding their CD22(Y822) phosphorylation and showed significant enhanced expression of p-CD22 after 15 and 30 min anti-IgM/IgG activation in CD27- SLE B cells. B cells were gated as CD3-, CD14-, CD20+ (data are represented as mean±SD; Mann Whitney U-test; **p<0.01).

4.1.2 Normal recruitment of SHP-1 and CD22 to the activated BCR complex in B cells of SLE patients

After BCR activation several kinases and co-receptors are recruited to the cytosolic part of the plasma membrane to form functionally competent signaling domains (lipid rafts)/ microclusters which modulate the B cell response by enhancing or in case of CD22 dampening the signal transduction (62, 64) by influencing the BCR activation threshold (79, 83). In order to gain further insights into the functional relevance of CD22 expression in SLE B cells, the localization of CD22 and SHP-1 was analyzed upon BCR activation.

Confocal microscopy studies addressed the co-localization of CD22, SHP-1 and the BCR after activation. These studies revealed a strong co-localization (co-localization coefficient >0.5) of these molecules after stimulation indicating a recruitment of SHP-1 to CD22 and CD22 to the activated BCR complex (**Figure 4-3**). In controls the co-localization of CD22 with the BCR was 0.38±0.16 without and 0.61±0.21 with BCR activation, in SLE patients 0.37±0.23 without and 0.61±0.17 with BCR stimulation. Similar results were obtained for the recruitment of SHP-1 to CD22. Control B cells showed an increase of the co-localization coefficient from 0.38±0.14 to 0.53±0.17. In SLE B cells the co-localization was 0.40±0.21 before and 0.57±0.19 after BCR engagement. However, no significant differences between HD and SLE B cells could be observed (mean±SD; **Figure 4-3B**).

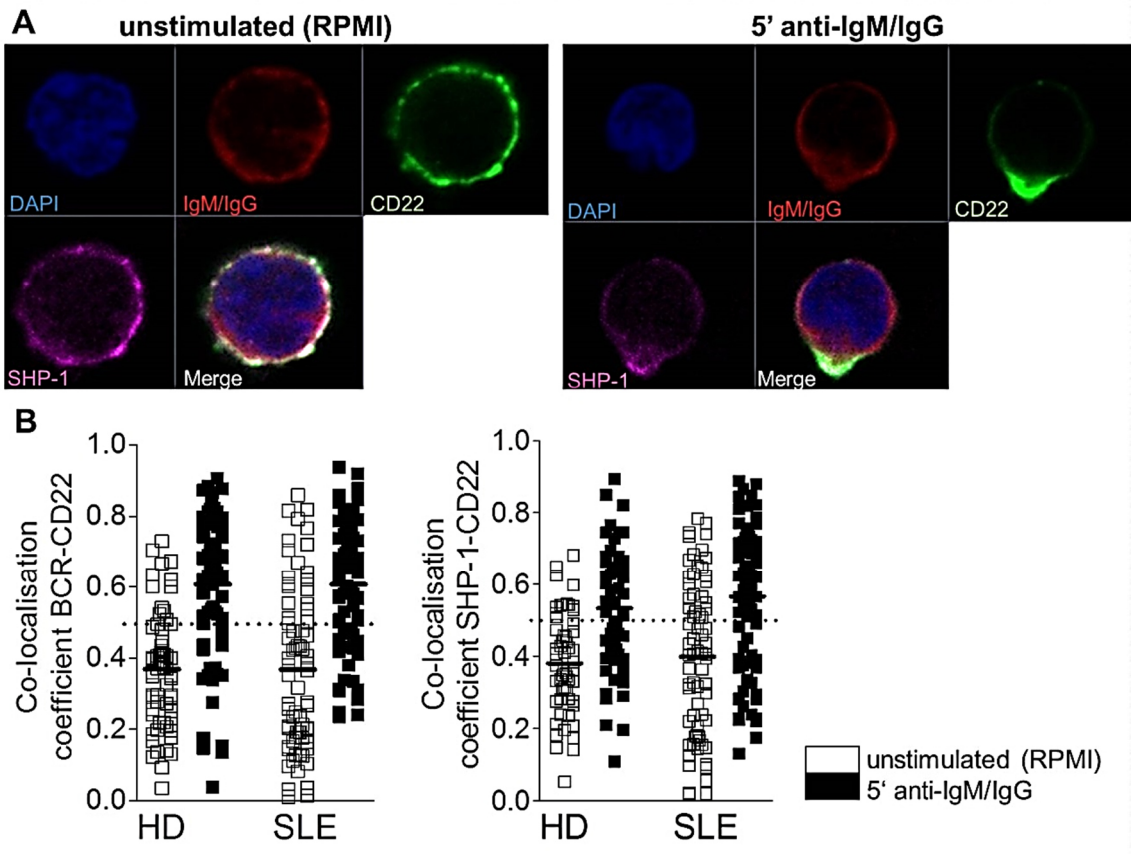

Figure 4-3: SLE B cells showed no disturbed co-localization of SHP-1 with CD22 or CD22 with the BCR after IgM/IgG activation. Purified B cells from SLE patients and HD were stimulated with RPMI (control) or anti-IgM/IgG for 5 min and stained **(A)** for IgM/IgG (red), CD22 (green) and SHP-1 (magenta). DAPI (blue) was used to stain the nucleus (original magnification: 630x). **B,** SLE compared to HD B cells were analyzed regarding their co-localization coefficient (merge) of CD22 with the BCR or SHP-1 with CD22 after RPMI (open square) or anti-IgM/IgG stimulation (filled square) revealing no differences between HD and SLE patients. Each symbol represents an individual B cell of 3 SLE and 3 HD; horizontal lines show the mean, Wilcoxon's signed rank test. Co-localization coefficient over 0.5 (dotted line) reflects co-localization between the two molecules analyzed.

This data indicate that the enhanced activation of CD22 upon BCR engagement in CD27- SLE B cells compared to HD is more likely due to a higher expression of CD22 and reduced ratio of CD19/CD22 in these cells than a disturbed signalosome formation and recruitment of CD22 and SHP-1 to the activated BCR complex.

4.1.3 Disturbed BCR response in patients with SLE

4.1.3.1 Reduced BCR response regarding Syk in B cells of SLE patients compared to HD

After BCR engagement a complex and tightly regulated signaling network becomes activated including the kinase Syk and further downstream PLC-γ2 but also Akt, a kinase which plays a crucial role in regulating the cell survival. To dissect potential implications of the enhanced CD22 phosphorylation of CD27- SLE B cells on the BCR

signaling, a comprehensive analysis on the phosphorylation of the BCR downstream key kinase Syk (Y352 and Y348), PLC-γ2 (Y759) and Akt on serine (S) 473 as well as on the intracellular Ca^{2+} mobilization upon BCR activation was performed (**Figure 4-4** and **Figure 4-6**).

At all-time points studied, total CD20+ B cells from SLE patients showed a reduced Syk(Y352) phosphorylation compared to HD (**Figure 4-4A, B**). After 5 min of anti-IgM/IgG stimulation (phosphorylation maximum of Syk(Y352)), CD20+ B cells from HD showed a p-Syk MFI of 2890±1636, while the p-Syk MFI of SLE CD20+ B cells was substantially lower (1670±402; mean±SD, *p<0.05). This difference was even more substantial when the phosphorylation efficiency of Syk in SLE and HD B cells was analyzed. Therefore, the ratio of the MFI of p-Syk and Syk was determined (**Figure 4-4B**). After 5 min BCR activation, B cells from HD displayed a MFI ratio of p-Syk(Y352)/Syk of 0.46±0.18 compared to the ratio of SLE B cells with 0.26±0.07 (mean±SD, **p<0.01, *p<0.05). This significantly lower ratio of SLE B cells was found at all-time points consistent with a globally reduced Syk phosphorylation efficiency in SLE.

Further analysis dissected CD20+ B cells into conventional CD27- naïve and CD27+ memory B cells. It has been shown that the thresholds of BCR dependent activation differs between these two B cell subsets (179) (**Figure 4-4C**). Interestingly, HD but not SLE patients showed stronger p-Syk(Y352) MFI values upon BCR stimulation in CD27+ B cells compared to CD27- B cells. CD27+ B cells from HD showed a p-Syk MFI of 3556±1744 and CD27- of 2731±1656 after 5 min of BCR stimulation, respectively (mean±SD, **p<0.01).

In contrast, CD27+ B cells from SLE patients showed only a modest increase in p-Syk MFI of 1689±600, whereas CD27- B cells developed a p-Syk MFI of 1704±483 (mean±SD, ns=1.00). The distinct p-Syk response of CD27+ versus CD27- B cells seen in controls was not detectable in B cells from SLE patients.

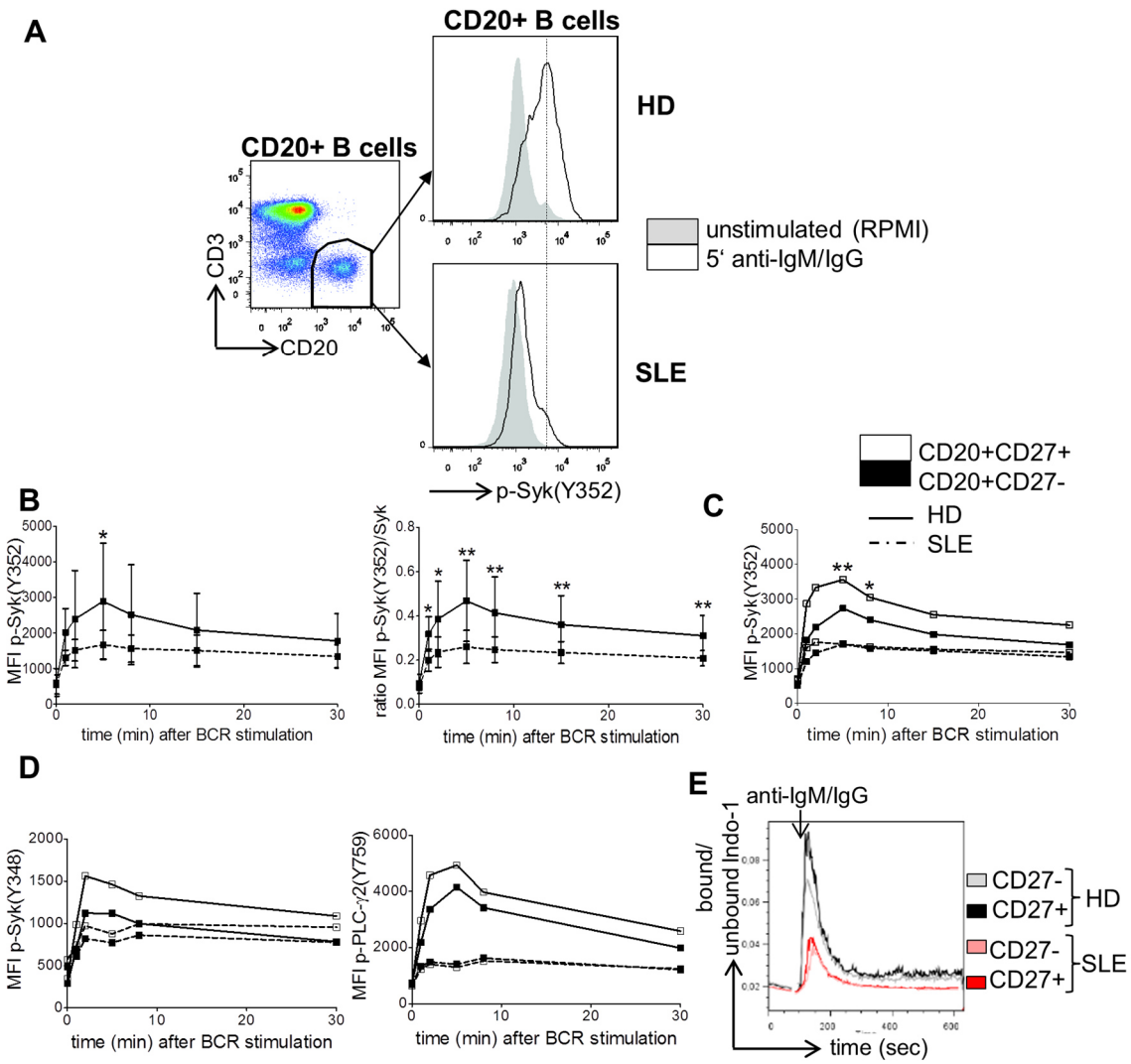

Figure 4-4: Diminished Syk and PLC-γ2 phosphorylation as well as a reduced Ca^{2+} influx upon BCR activation is a characteristic of SLE B cells. A, Representative histograms comparing the p-Syk(Y352) expression by CD20+ B cells from SLE patients and HD after 5 min RPMI (control) or anti-IgM/IgG stimulation. **B,** PBMC from SLE patients (n=9) and HD (n=10) were stimulated with anti-IgM/IgG for 0, 1, 2, 5, 8, 15 and 30 min. Comparative time kinetics of CD20+ B cells from SLE patients (dotted line) and HD (solid line) regarding their Syk phosphorylation (Y352) as well as the MFI ratio of p-Syk(Y352)/total Syk are shown (data are represented as mean±SD, *p<0.05 and **p<0.01 by Mann Whitney U-test). **C,** Phosphorylation of Syk was analyzed for CD27+ (open square) and CD27- (filled square) B cells, respectively (data are represented as mean±SD, *p<0.05 and **p<0.01 by Mann Whitney U-test). **D,** PBMC from 5 SLE patients and 5 HD were stimulated with anti-IgM/IgG for 0, 1, 2, 5, 8 and 30 min. CD27+ (open square) and CD27- (filled square) B cells of SLE patients and controls were analyzed regarding their Syk(Y348) and PLC-γ2(Y759) phosphorylation (data are represented as mean). **E,** Representative time kinetic of the intracellular Ca^{2+} concentration of Indo 1-AM-loaded CD27+ and CD27- B cells after BCR stimulation (SLE patients: CD27+=red; CD27- =light red; HD: CD27+=black; CD27-= grey; n=3). This data disclose a disturbed BCR response in SLE B cells and showed comparable responses of CD27- and CD27+ SLE B cells compared to HD.

Interestingly, analyzing another important Syk phosphorylation site (Y348) revealed similar results as found for Y352 (**Figure 4-4D**). CD27+ as well as CD27- SLE B cells displayed reduced phosphorylation kinetics regarding Syk(Y348) and diminished phosphorylation responses between these two B cell subsets compared to controls. Here, p-Syk(Y348) revealed a MFI of 876±433 for CD27+ and 772±381 for CD27- SLE B cells

after 5 min of BCR stimulation. The mean MFI value for HD was substantially higher (CD27+: 1461±381, CD27- 1119±293; mean±SD).

To analyze disturbed downstream signaling events in SLE patients, the phosphorylation of PLC-γ2(Y759) as well as the Ca^{2+} influx were studied after BCR stimulation. Comparable to the phosphorylation response of Syk, PLC-γ2 displayed reduced phosphorylation amplitude after stimulation with anti-IgM/IgG (**Figure 4-4D**). The control group showed MFI values of 4933±2734 in CD27+ and of 4152±3051 in CD27- B cells compared to SLE patients with a MFI of 1300±453 in CD27+ and of an even higher value of 1406±481 in CD27- B cells. In addition, a reduced BCR-induced Ca^{2+} mobilization in both CD27+ and CD27- B cell subsets in patients with SLE was detected compared to B cells from HD (**Figure 4-4E**). Interestingly and consistent with the findings for p-Syk and p-PLC-γ2, no differences in BCR-induced Ca^{2+} mobilization between CD27- and CD27+ SLE B cells were observed.

4.1.3.2 Isotype independent reduced Syk activation after BCR stimulation in patients with SLE

A recently published paper investigated different phosphorylation patterns after BCR activation in switched (IgG+) versus pre-switch (IgM+) memory B cell showing a reduced BCR response in pre-switch IgM+ memory B cells (75). Therefore, the distribution of IgM+ and IgG+ memory B cells in SLE patients compared to HD as well as the Syk phosphorylation after IgM activation was analyzed. Since no differences between the percentage of IgM+ and IgG+ B cells within the CD27+ B cell subset of SLE and HD has been found (HD: IgM+ 49.2±8.6%, IgG+ 28.5±12.4%; SLE: IgM+ 58.6±16.3%, IgG+ 23.6±10.9; mean±SD; **Figure 4-5A**), the reduced Syk phosphorylation in SLE CD27+ B cell is not related to an enhanced occurrence of less responsive IgM+ memory B cells in these patients and reflect rather an intrinsic molecular defect. Stimulation with only anti-IgM revealed a reduced Syk phosphorylation in CD27+IgM+ memory B cells of SLE patients compared to controls (**Figure 4-5B**). CD27+ SLE B cells had p-Syk MFI values after 5 min BCR activation of 2957±883 compared to 3941±1075 of healthy controls indicating a global reduction of Syk activation after BCR stimulation in CD27+ memory B cells of SLE patients independent of their isotype.

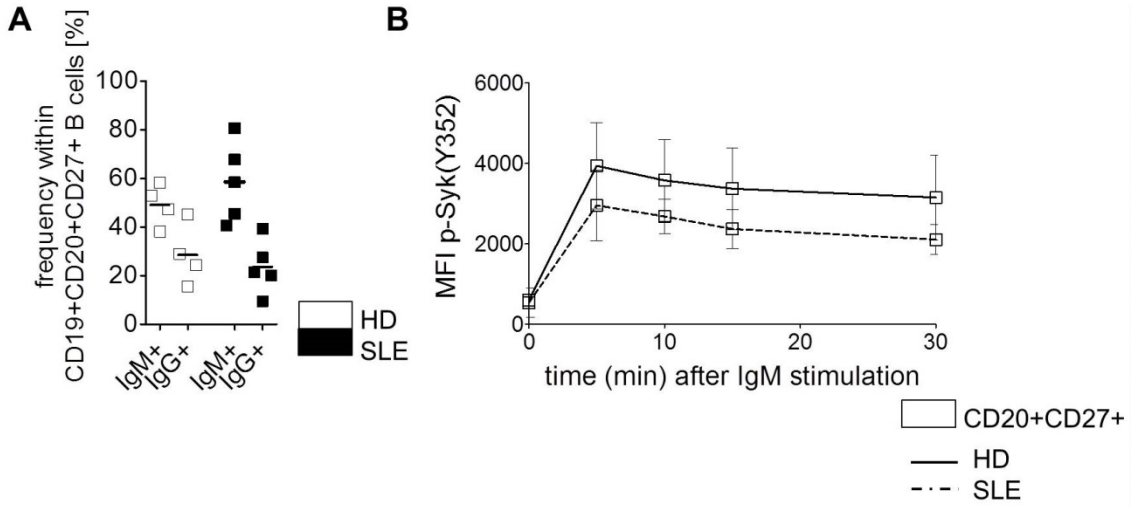

Figure 4-5: Comparable frequencies of IgM+ and IgG+ memory B cells in SLE and HD but reduced Syk phosphorylation kinetics in IgM+ memory B cells of SLE patients after BCR activation. A, The frequency of CD27+IgM+ and CD27+IgG+ memory B cells from SLE (filled square) compared to HD (open square), respectively are shown demonstrating no differences between HD and SLE patients regarding the occurrence of IgM or IgG memory B cells. Each symbol represents an individual subject of 5 SLE and 4 HD; horizontal lines show the mean, Mann Whitney U-test. **B,** PBMC from 5 SLE patients and 6 HD were stimulated with anti-IgM for 0, 5, 10, 15 and 30 min. CD27+ B cells (open square) of SLE patients (dotted line) and controls (solid line) were analyzed regarding their Syk(Y352) phosphorylation revealing also in CD27+IgM+ SLE B cells a reduced BCR response (data are represented as mean).

4.1.3.3 Hyperactive Akt phosphorylation and imbalanced p-Syk/p-Akt ratio on B cells of SLE patients

In contrast, phosphorylation of Akt(S473) after BCR activation unfolded a significantly increased Akt activation in SLE B cells compared to HD (**Figure 4-6A**). After 5 min BCR activation CD27- B cells from SLE patients showed Akt phosphorylation MFI values of 1786±395 compared to 1015±583 in HD (mean±SD, *p<0.05). After 15 min anti-IgM/IgG stimulation (phosphorylation maximum of Akt(S473)) an enhanced response has been even observed in both CD27 B cell subsets (HD: CD27+ 1382±598 MFI, CD27- 961±534 MFI; SLE: CD27+ 2151±804 MFI, CD27- 1848±505 MFI; *p<0.05, **p<0.01). After 30 min of BCR stimulation CD27- SLE B cells revealed enhanced p-Akt MFI values (SLE: 1747±471, HD: 921±523; *p<0.05) demonstrating a higher Akt phosphorylation in patients with SLE especially in CD27- B cells. Since an enhanced activation of Akt in SLE patients was observed, a global or general unresponsiveness of SLE B cells related to chronic *in vivo* activation, therapeutic interventions or disease activity is rather unlikely. In line with this, no correlation between the BCR response regarding p-Syk or p-Akt and the treatment or the SLE disease activity index (SLEDAI) of these patients has been observed (data not shown).

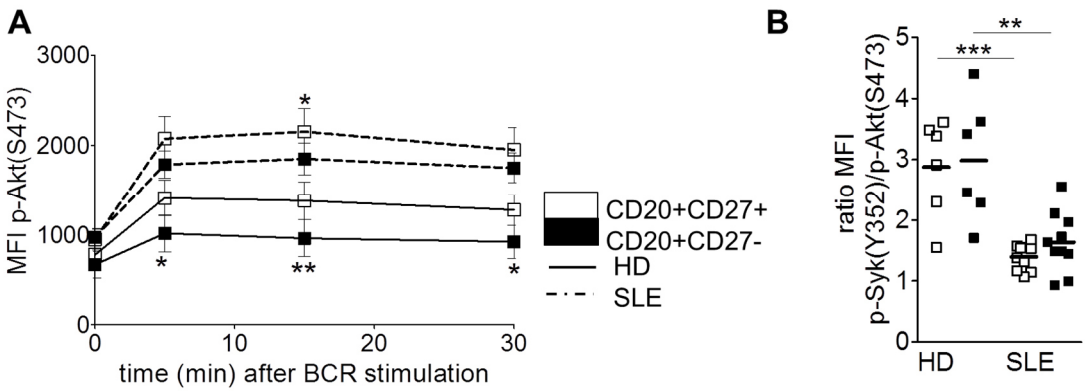

Figure 4-6: Enhanced activation of the pro-survival kinase Akt and disturbed balance between the p-Syk and p-Akt pathway in CD27+ and CD27- SLE B cells after BCR activation. A, PBMC from SLE patients (dotted, n=7) and HD (solid, n=8) were stimulated with anti-IgM/IgG for 0, 5, 10, 15 and 30 min and the phosphorylation of Akt at serine (S) 473 in CD27+ (open square) and CD27- (filled square) B cells were analyzed showing enhanced phosphorylation kinetics in CD27+ and CD27- SLE B cells. B cells were gated as CD3-, CD14-, CD20+, CD27+ or CD27- (data are represented as mean±SEM, **p<0.01, *p<0.05 by Mann Whitney U-test). **B,** The ratio shown here of p-Syk(Y352) (5 min) and p-Akt(S473) (15 min) was analyzed in CD27+ (open square) and CD27- (filled square) B cells from SLE compared to HD demonstrating a disturbed balance between this two kinases analyzed. Each symbol represents an individual subject of 10 SLE and 6 HD; horizontal lines show the mean, Mann Whitney U-test; ***p<0.001, **p<0.01).

Subsequent studies addressed the balance of Syk and Akt activation by using the ratio of p-Syk/p-Akt, since the B cell fate is determined by the equilibrium of different signaling pathways (56). A significant decreased p-Syk/p-Akt ratio in CD27+ and CD27- B cells was found in patients with SLE clearly demonstrating a shifted balance towards the pro-survival kinase Akt (**Figure 4-6B**). Here CD27+ B cells of HD showed a p-Syk/p-Akt ratio of 2.88±0.81 and CD27- B cells of 2.99±1.00. SLE patients had a ratio of 1.40±0.21 for CD27+ and 1.63±0.49 for CD27- B cells (mean±SD, **p<0.01, ***p<0.001), respectively. No significant differences were observed between CD27+ and CD27- B cells of HD and SLE patients, exhibiting a comparable balance of Syk and Akt activation in these two B cell subsets. Interestingly, in both B cell subsets (CD27+ and CD27- B cells) a striking positive correlation between the ratio of CD19/CD22 expression and the ratio of p-Syk/p-Akt has been found (CD27+ r=0.6400, CD27- r=0.5742, *p<0.05, **p<0.01; **Figure 4-7**) although the CD19/CD22 ratio was only significantly decreased in SLE CD27- B cells. This data indicate that the balance of the BCR co-receptors CD19 and CD22 and the balance between Syk and Akt pathways are strongly interdependent.

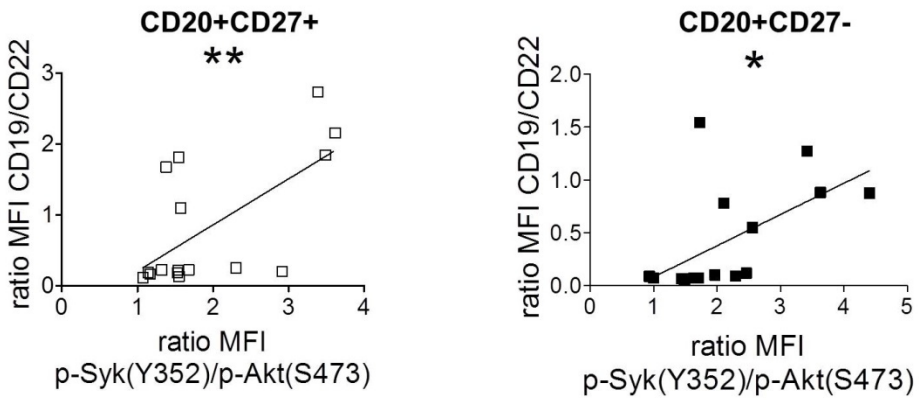

Figure 4-7: The balance of CD19/CD22 expression correlates with the balance of Syk and Akt phosphorylation after BCR activation. Pearson's correlation analysis of the ratio of CD19/CD22 expression and the MFI ratio of p-Syk (5 min)/p-Akt (15 min) upon anti-IgM/IgG stimulation in CD27+ (open square, r=0.6400) and CD27- B cells (filled square, r=0.5742) is shown. This data indicate that the expression of the BCR co-receptors CD19 and CD22 directly regulates the activation strength of the two signaling pathways in CD27+ as well as in CD27- B cells. Each symbol represents an individual subject of 10 SLE and 6 HD; **p<0.01, *p<0.05.

4.1.4 Increased tyrosine phosphatase activity in patients with SLE

It has been shown earlier that a balanced kinase/phosphatase (e.g. Syk/SHP-1) equilibrium plays a crucial role for normal B cell signaling, development and function (90). Based on the observation, that SLE B cells exhibited a reduced Syk phosphorylation on tyrosine 352, tyrosine 348 and on PLC-γ2 tyrosine 759 but enhanced Akt phosphorylation on serine 473, the tyrosine and serine/threonine phosphatase activity was analyzed in phosphate free B cell lysates. Specific phosphorylated peptides (Tyr-1, Tyr-2 and Ser/Thr) were used as substrate to assess the tyrosine and serine/threonine phosphatase activities.

Dephosphorylation of the peptides by phosphatases in the B cell lysates of SLE patients and controls including the tyrosine phosphatase SHP-1 led to an increase of free phosphate. Therefore, a high concentration of free phosphate is consistent with an elevated phosphatase activity in the corresponding B cell lysate. As shown in **Figure 4-8A** a higher phosphate concentration (pMol free phosphate/ $0.5x10^5$ B cells) after incubation of the Tyr-1 with the B cell lysate from SLE patients was measured resulting in an enhanced tyrosine phosphatase activity in these patients (HD: Tyr-1 1549±198; SLE: Tyr-1 3140±1550 mean±SD, *p<0.05). For Tyr-2 and Ser/Thr no differences have been seen between HD and SLE patients.

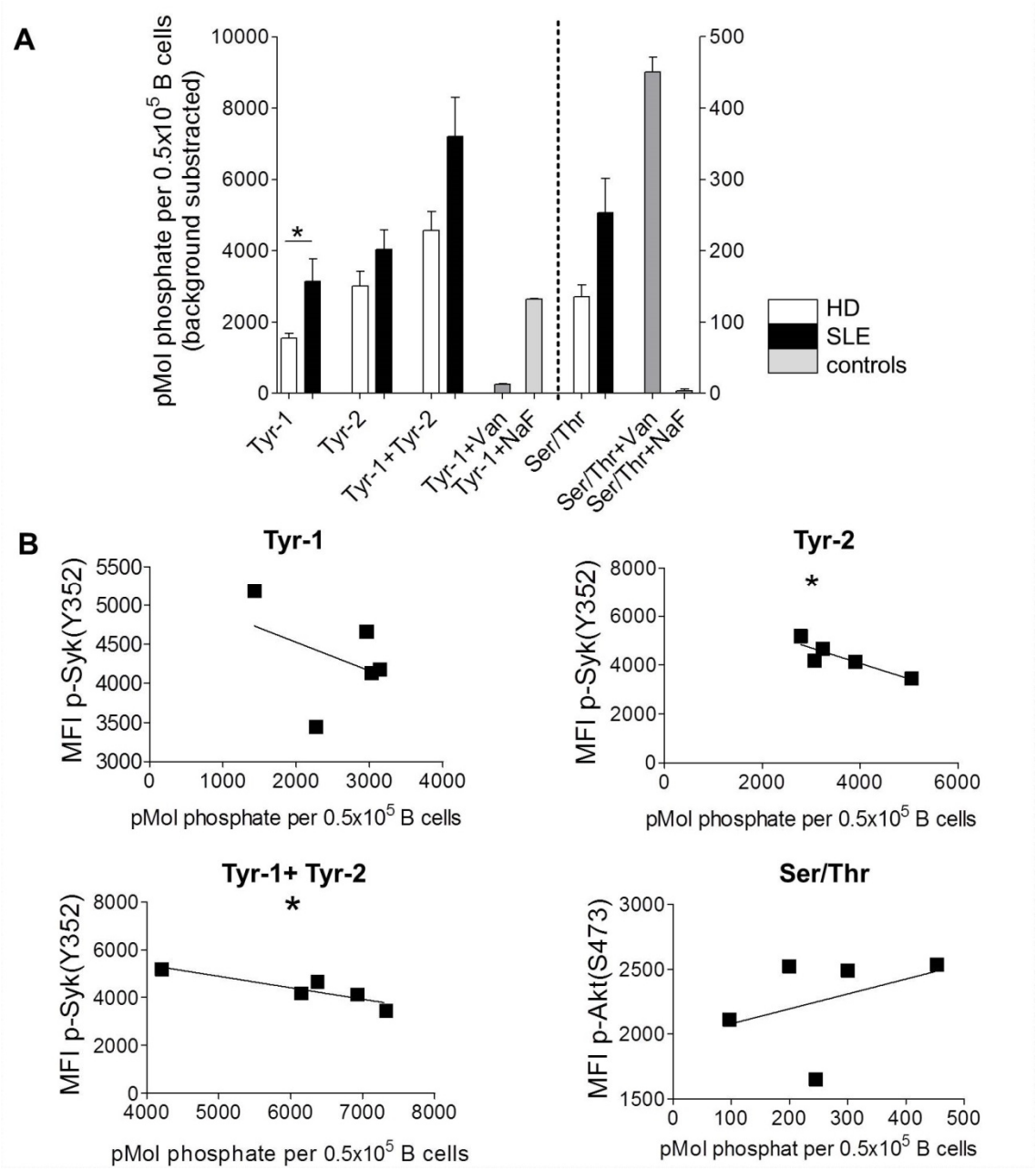

Figure 4-8: Enhanced tyrosine phosphatases activity which directly influenced the phosphorylation of Syk(Y352) after BCR activation in B cells from SLE patients. A, Tyrosine (Tyr-1 and Tyr-2) and serine/threonine (Ser/Thr) phosphatase activity was measured in phosphate free B cell lysates from 6 SLE patients (filled bar) and 5 HD (open bar) represented by the concentration of cleaved phosphate in pMol/0.5x10^5 B cells. Background was subtracted. Specificity was analyzed by phosphatase inhibitors (sodium orthovanadate (Van)= inhibits tyr phosphatases; sodium fluoride (NaF)= inhibits ser/thr phosphatases, grey bar; data are represented as mean±SD, *p<0.05 by Mann Whitney U-test. **B,** Pearson's correlation between the p-Syk(Y352) or p-Akt(S473) MFI and the free phosphate per 0.5x10^6 B cells in pMol in patients with SLE (n=5); Tyr-2 r=-0.8856, Tyr-1+Tyr-2 r=-0.8882, *p<0.05.

To validate the specificity, the phosphatase inhibitors sodium orthovanadate (Van; tyrosine phosphatase inhibitor) or sodium fluoride (NaF; serine/threonine phosphatase inhibitor) were added and could demonstrate a specific inhibition of the phosphatase activity (Tyr-1+Van 250±32, Tyr-1+NaF 2631±42; Ser/Thr+Van450±30, Ser/Thr+NaF3±4; mean±SD; **Figure 4-8A**).

In addition, for the phosphorylated peptide Tyr-2 and the combination of Tyr-1+Tyr-2 a significant correlation between the concentration of free phosphate and the phosphorylation of Syk(Y352) after 5 min BCR activation was determined (Tyr-2 r=-0.8856, Tyr-1+Tyr-2 r=-0.8882, *p<0.05; **Figure 4-8B**). This data revealed a negative correlation between the tyrosine phosphatase activity and the BCR response regarding Syk but not between the serine/threonine tyrosine phosphatase activity and the activation of Akt after BCR engagement. Since no differences between SLE and controls regarding their phosphatase activity specific for Tyr-2 and Ser/Thr peptides were measured, a general abnormality of phosphatase activity in SLE patients can be excluded.

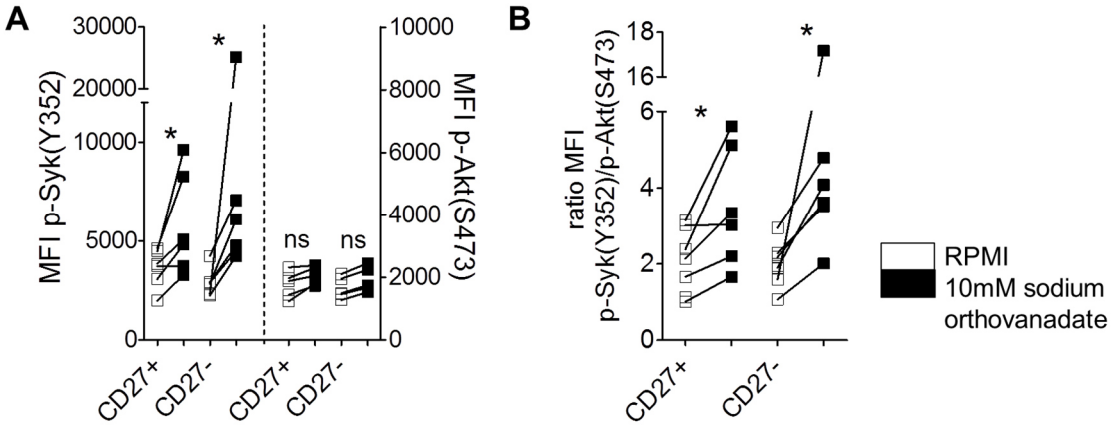

Figure 4-9: Inhibition of tyrosine phosphatase activity led to an enhanced Syk phosphorylation and shifted balance of p-Syk/p-Akt towards the tyrosine kinase Syk in SLE B cells. Syk(Y352) and Akt(S473) phosphorylation **(A)** and the ratio of p-Syk/p-Akt **(B)** was measured in BCR activated B cells with (filled square) and without pre-treatment (open square) with 10mM sodium orthovanadate from 6 SLE patients; each symbol represents an individual subject; *p<0.05 by Wilcoxon's signed rank test.

Furthermore, treatment of SLE PBMC with sodium orthovanadate which inhibits specific the tyrosine phosphatase activity significantly enhanced the phosphorylation of Syk(Y352) after BCR activation and shifted the ratio of p-Syk/p-Akt towards Syk phosphorylation (**Figure 4-9**). The MFI of p-Syk increased from 3623±979 to 5804±2565 in CD27+ B cells and from 2910±718 to 8624±8144 in CD27- B cells (mean±SD, *p<0.05). As a control, no difference between the phosphorylation of Akt(S473) has been observed after 15 min anti-IgM/IgG stimulation with or without sodium orthovanadate pre-treatment (**Figure 4-9A**). The ratio of p-Syk/p-Akt shifted in CD27+ B cells from 2.07±0.85 to 3.49±1.58 and in CD27- B cells from 1.98±0.59 to 5.87±5.6 (mean±SD, *p<0.05; **Figure 4-9B**).

These observations raise the interesting possibility that the disturbed CD22/CD19 balance of SLE B cells is related to an enhanced activity of tyrosine phosphatases and

leads to a reduced Syk and PLC-γ2 phosphorylation with disturbed Ca^{2+} mobilization after BCR activation. Furthermore, it indicates that the disturbed tyrosine phosphatase activity in SLE patients controls the balance between the p-Syk and p-Akt signaling pathway.

4.1.5 Imbalanced BCR signaling in SLE patients led to an increased survival of SLE B cells

To investigate the consequences of the dysbalanced p-Syk/p-Akt ratio towards the pro-survival kinase Akt, the survival of anti-IgM/IgG stimulated B cells using the frequency of viable (AnnexinV and PI double negative) cells was analyzed as a function of the BCR response. For this purpose an AnnexinV/PI staining was performed on 60 min anti-IgM/IgG stimulated compared to unstimulated B cells (**Figure 4-10A**). The viability of CD27+ as well as CD27- B cells after 60 min BCR activation appeared to be significant correlated with the ratio of p-Syk/p-Akt observed after 5 min BCR stimulation for p-Syk and 15 min BCR stimulation for p-Akt (phosphorylation maximum of both kinases) (**Figure 4-10B**). This data showed a direct impact of the shifted balance towards Akt and the enhanced survival of CD27+ and CD27- B cells. Therefore, the imbalanced BCR signaling towards Akt activation in SLE patients may result in a survival advantage of (auto)-reactive B cells.

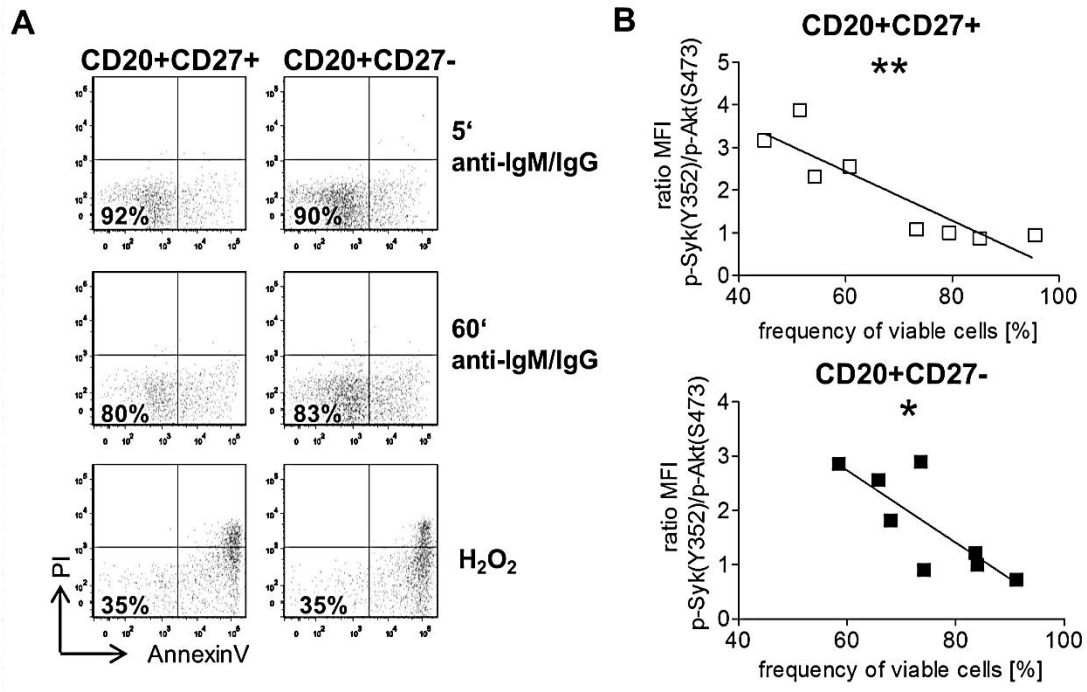

Figure 4-10: Shifted balance towards Akt phosphorylation enhances the survival of B cells. A, Representative dot plots are shown for the gating strategy of viable CD27+ and CD27- B cells (AnnexinV-/PI-) after 5 and 60 min anti-IgM/IgG stimulation. H_2O_2 treated cells were used as AnnexinV/PI positive control. **B,** Pearson's correlation between the ratio of p-Syk/p-Akt and the frequency of viable cells after 60 min anti-IgM/IgG stimulation in CD27+ (open

square) and CD27- (filled square) B cells indicating that enhanced phosphorylation of Akt is related with enhanced survival. Each symbol represents an individual subject of 4 SLE and 4 HD; *p<0.05, **p<0.01.

4.1.6 Disturbed BCR response in SLE patients is independent of an *in vivo* pre-activation

To determine to which extent an *in vivo* pre-activation could lead to this hyporesponsive phenotype in SLE patients regarding Syk, a comprehensive analysis on whole blood CD27+ and CD27- B cells from HD and SLE patients regarding their expression of CD80 and CD86 was performed (**Figure 4-11**). The co-stimulatory molecules CD80 and CD86 play an important role during immune activation and can be used as activation marker for *in vivo* activation (180).

As shown in **Figure 4-11B**, a significant increased frequency of CD27+CD86+, CD27-CD86+ as well as CD27-CD80+ B cells in SLE patients compared to HD was observed as it has been described previously (181).

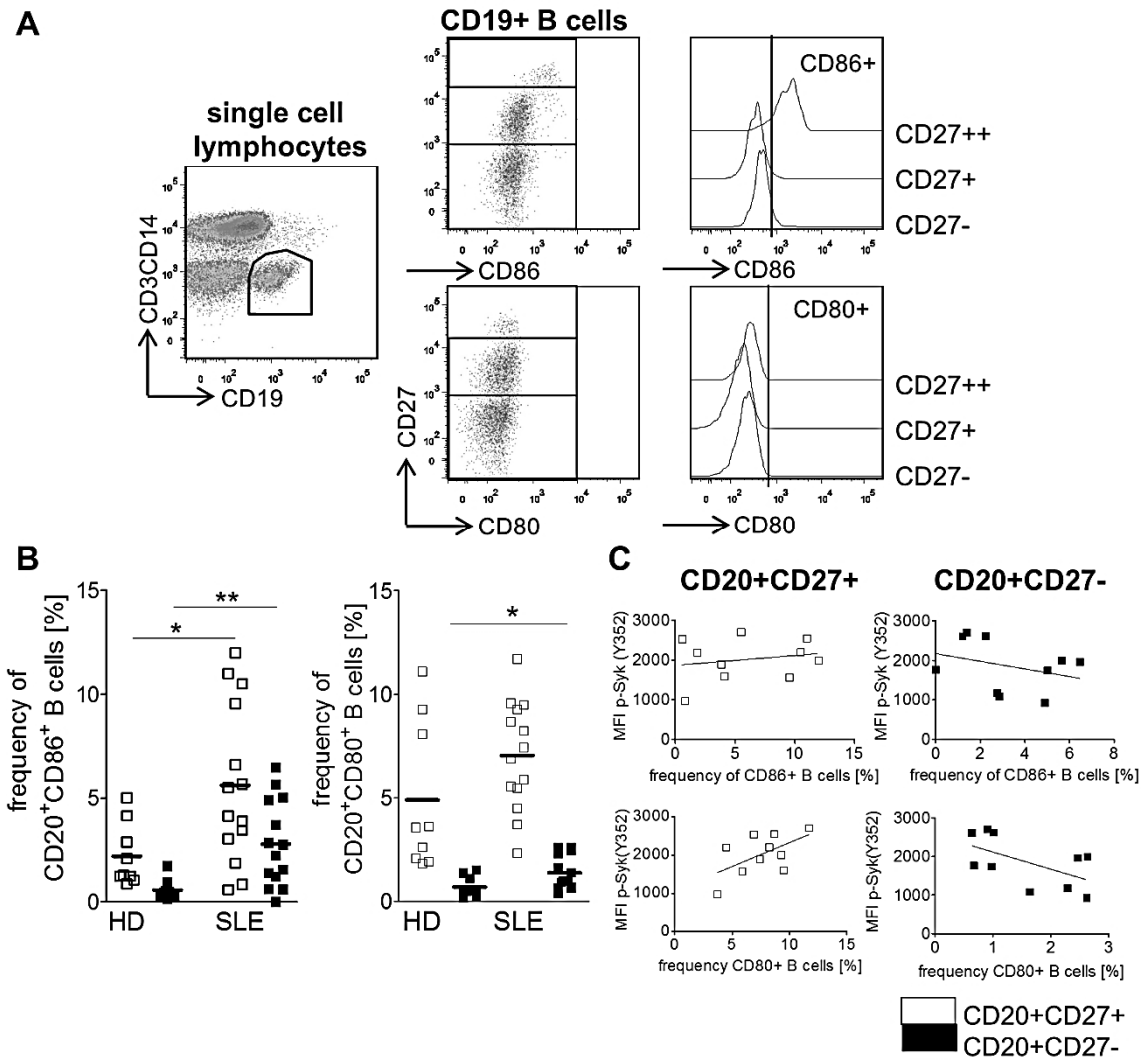

Figure 4-11: The aberrant BCR response of SLE B cells is independent of an *in vivo* pre-activation represented by the occurrence of CD80+ and CD86+ B cells. A, Representative dot plots and histograms are shown for the gating strategy of CD86+ and CD80+ B cells within CD19+CD27+ and CD19+CD27- B cell subsets. **B,** Whole blood CD27+ (open square) and CD27- (filled square) B cells obtained from 14 SLE patients and 9 HD were analyzed regarding their frequency of CD86 and CD80 positive cells. Each symbol represents an individual subject; horizontal lines show the mean; **p>0.01, *p<0.05 by Mann-Whitney U test. **C,** Pearson's correlation between the MFI of p-Syk in CD27+ (open square) and CD27- (filled square) SLE B cells after 5 min of anti-IgM/IgG stimulation and the frequency of CD86+ and CD80+ B cells showed no correlation between the strength of Syk(Y352) activation and incidence of CD80+ or CD86+ B cells. Each symbol represents an individual subject of 10 SLE patients.

The frequency of CD27+CD86+ B cells of SLE patients was 5.6±3.8% compared to 2.2±1.5% in CD27+ B cells of HD (mean±SD, *p<0.05). Within the CD27- compartment 2.8±2.1% in SLE patients but only 0.6±0.5% in HD were CD86+ (mean±SD, **p<0.01). Regarding CD80+ B cells, only CD27- SLE B cells showed significant enhanced frequencies (SLE: 1.4±0.8%, HD: 0.7±0.5; mean±SD, *p<0.05). This data indicate a higher pre-activation of SLE B cells *in vivo*. However, comparing the frequency of CD86+ and CD80+ B cells with the phosphorylation of Syk after BCR activation, no correlation has been observed (**Figure 4-11C**).

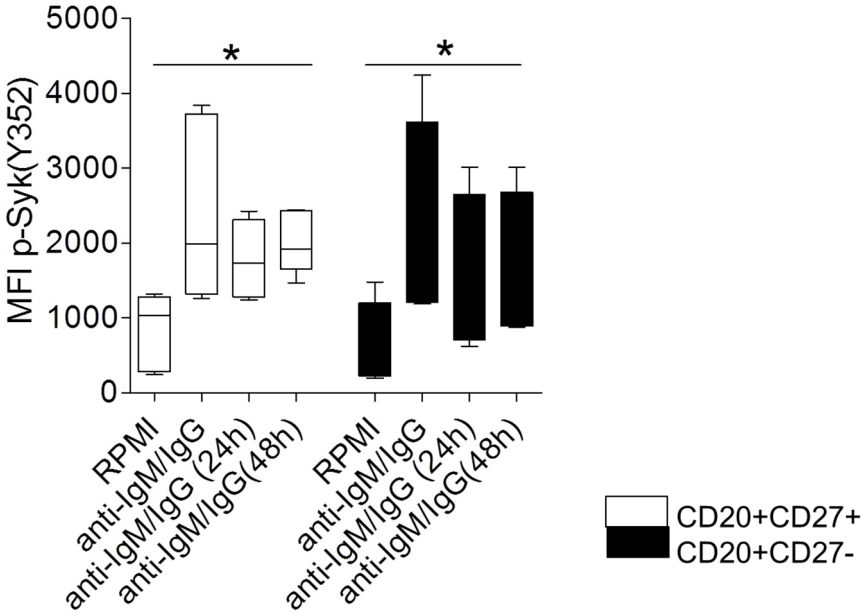

Figure 4-12: The *in vivo* environment of SLE patients had no impact on the Syk phosphorylation in response to BCR ligation. PBMC from 7 SLE patients were stimulated with anti-IgM/IgG for 5 min immediately ex *vivo* (0 h) or after a 24 h and 48 h *in vitro* resting phase in RPMI/10% FCS. Syk(Y352) phosphorylation of CD27+ (open bars) and CD27- (filled bars) B cells was analyzed; data are represented as box and whiskers min to max. Lines inside the boxes represent the median; *p<0.05 by Wilcoxon matched pair test.

In addition, isolated SLE PBMC were incubated for 24 and 48 h *in vitro* to analyze if removing the *in vivo* environment could rescue the BCR responsiveness in SLE B cells regarding Syk activation. Interestingly, an additional resting phase of 24 h and 48 h did not led to a normalization of the Syk phosphorylation after BCR stimulation in SLE CD27+ and CD27- B cells. In detail, the MFI of p-Syk(Y352) at day 0 was 1988±1108 for CD27+ and 2317±1150 for CD27- B cells compared to 1732±1100 for CD27+ and 1860±1345 for CD27- B cells after 24 h and 1918±939 (CD27+ B cells) and 1860±1261 (CD27- B cells) after 48 h *in vitro* resting (median±SD; **Figure 4-12**).

This data indicate that the expression of CD86+ and CD80+ B cells and therefore a pre-activation *in vivo* in SLE patients or the *in vivo* environment with enhanced levels of free dsDNA, pro-inflammatory cytokines and auto-Ab levels (120, 182-184) appears to be independent from the hyporesponsive BCR reaction regarding Syk and PLC-γ2 activation. Therefore, an intrinsic molecular defect in B cells of SLE patients is suggested.

4.2 Enhanced frequency of a unique Syk bright memory B cell subset in patients with SLE

4.2.1 Whole blood CD27- SLE B cells expressed high levels of Syk

Further studies compared the basal expression of Syk and p-Syk(Y352) in whole blood SLE versus HD B cells to dissect if a disturbed expression of Syk and p-Syk(Y352) could be involved in the reduced Syk phosphorylation response after BCR activation. As expected CD27+ memory B cells in HD as well as in SLE patients express higher MFI levels of Syk and p-Syk(Y352) compared to CD27- B cells (HD: CD27+ 2195±458, CD27- 1335±301; SLE: CD27+ 1884±355, CD27- 1261±363; mean±SD, ***p<0.001; **Figure 4-13A**).

However, comparing SLE patients with HD B cells showed a significant reduced expression of Syk in CD27+ memory SLE B cells (*p<0.05) and p-Syk in CD27- and CD27+ B cells (HD: CD27+ 456±132, CD27- 339±103; SLE: CD27+ 366±126, CD27- 272±113; mean±SD, *p<0.05). Therefore, a reduced basal expression of Syk and p-Syk(Y852) could be beside the enhanced tyrosine phosphatase activity and the disturbed CD19/CD22 balance responsible for the reduced Syk phosphorylation efficiency after BCR activation in SLE.

Interestingly, analyzing Syk within the CD27- B cell compartment revealed a unique Syk high expressing subpopulation in SLE patients. Comparing the frequency of CD27-Syk++ B cells between SLE patients, HD and other autoimmune diseases like RA and pSS showed a significantly enhanced frequency in patients with SLE (**Figure 4-13B**).

In detail, the frequency in HD of CD27-Syk++ was 6.4±4.0% with only 4 of 28 having a frequency over 10%, by contrast 20 out of 33 patients (*p<0.05 by Fisher`s exact test) showed a frequency of CD27-Syk++ B cells with a frequency of 16.1±14.1% (mean±SD, **p>0.01). RA patients and pSS patients did not show an increase of this population (8.6±4.5% (RA) and 10.1±8.0% (pSS)), except in individual cases, compared to HD. Thus, an overrepresented frequency of peripheral CD27-Syk++ B cells was very characteristic for patients suffering from SLE.

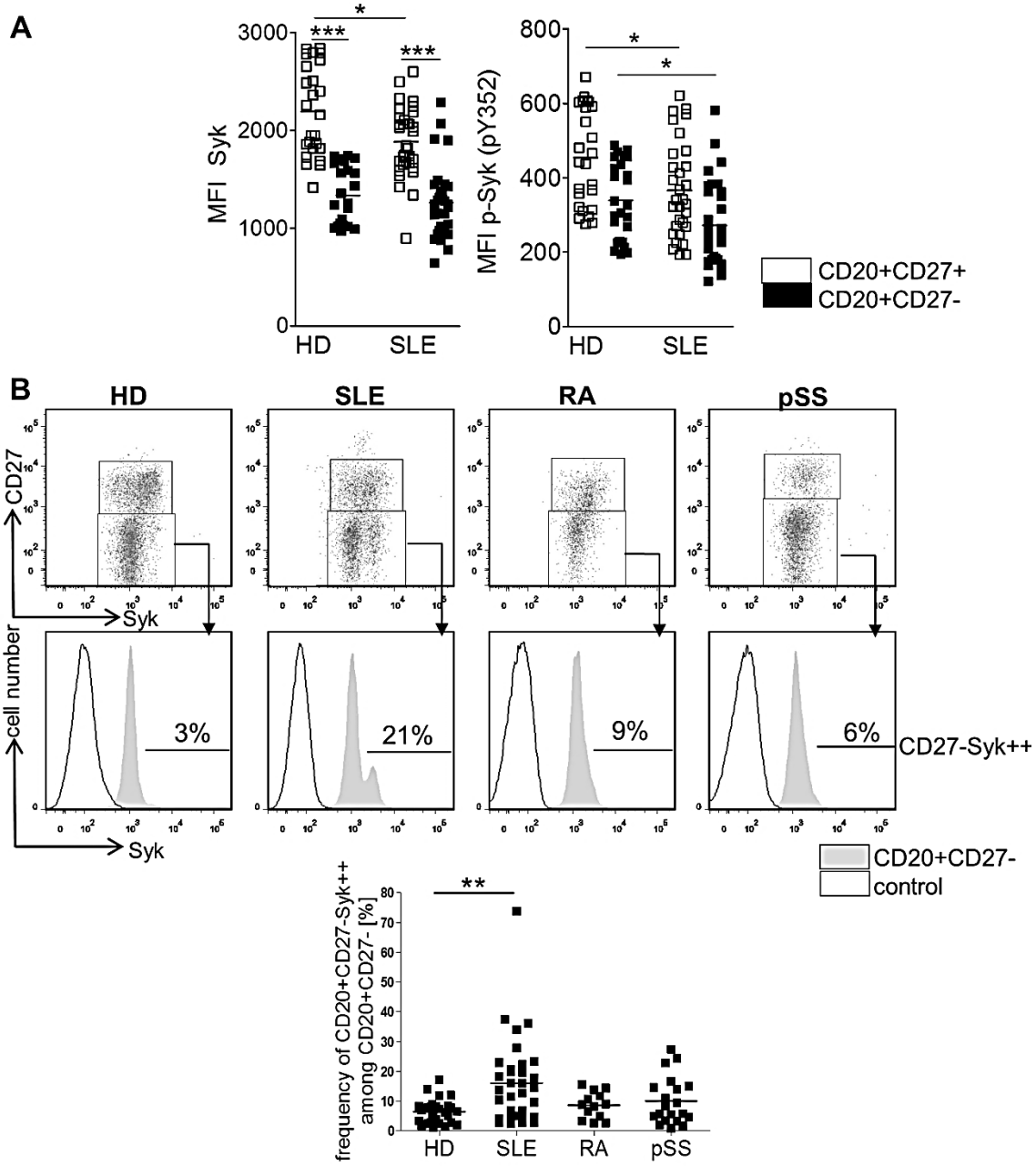

Figure 4-13: Peripheral blood of SLE patients revealed increased frequencies of a unique CD27-Syk++ B cell subset. A, The expression of Syk and p-Syk(Y352) in CD27- and CD27+ B cells was analyzed in whole blood from HD (n=25) and patients with SLE (n=33); *p<0.05 by Mann-Whitney U test. **B,** Representative histograms comparing Syk expression by CD27- B cells and T cells (negative control) are shown. Dot plot shows enhanced frequencies of CD27-Syk++ B cells in patients with SLE (n=33) compared to HD (n=28), rheumatoid arthritis patients (RA; n=13), or primary Sjögren's syndrome patients (pSS; n=20), respectively. Each symbol represents an individual subject; horizontal lines show the mean; **p<0.01 by Mann-Whitney U test.

Interestingly, analyzing the phosphorylation response of Syk and Akt after BCR stimulation revealed a memory like BCR response of CD27-Syk++ compared to CD27-Syk+ and CD27+ B cells. CD27-Syk++ B cells showed a p-Syk(Y352) MFI value of 2351±411 after 5 min of BCR stimulation which was comparable with CD27+ memory B cells (2391±595), while the phosphorylation of Syk in CD27-Syk+ B cells was only 1272±224. Similar results were obtained for p-Akt(S473) after 15 min BCR stimulation

(CD27-Syk++: 2849±140, CD27+ 2491±311, CD27-Syk+: 1965±200; mean±SD; **Figure 4-14A, B**).

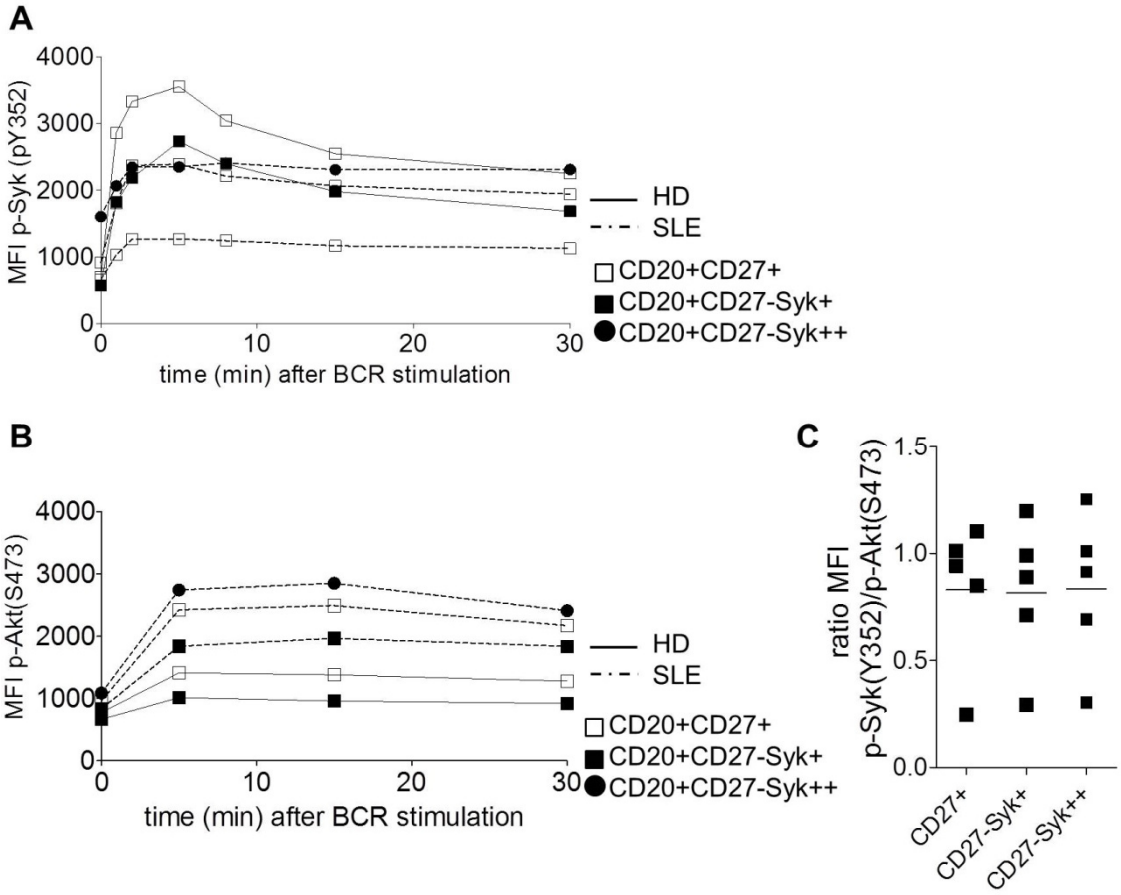

Figure 4-14: CD27-Syk++ B cells from SLE patients showed a memory-like BCR dependent phosphorylation kinetics. A, Time kinetics of CD27-Syk+ (filled square), CD27-Syk++ (filled circle), and CD27+ (open square) B cells from SLE patients (dotted, n=3) and HD (solid, n=11) are shown as mean. PBMC were stimulated with anti-IgM/IgG for 0, 1, 2, 5, 8, 15, and 30 min revealing similar phosphorylation kinetics of CD27-Syk++ compared to CD27+ SLE B cells but still reduced compared to HD B cells. **B,** PBMC from SLE patients were stimulated with anti-IgM/IgG for 0, 5, 15 and 30 min. Comparative time kinetics between CD27-Syk+ (filled square), CD27-Syk++ (filled circle), and CD27+ (open square) SLE (dotted line, n=3) and HD (solid line, n=8) B cells of the phosphorylation of Akt(S473) unfolded increased activation in CD27-Syk++ B cells. Data are represented as mean. **D,** The ratio of p-Syk(Y352) and p-Akt(S473) was analyzed in CD27-Syk+, CD27-Syk++, and CD27+ B cells from SLE patients disclosing no differences between the balance of p-Syk and p-Akt between the three B cell subsets analyzed. Each symbol represents an individual sample of 5 SLE patients; horizontal lines show the mean.

However, the phosphorylation kinetics of Syk after BCR stimulation in CD27-Syk++, CD27-Syk+ and CD27+ SLE B cells was still diminished compared to healthy controls. Noteworthy, comparing the phosphorylation ratio of p-Syk/p-Akt no differences between CD27-Syk++, CD27+ and CD27-Syk++ B cells have been seen. This data indicate a B cell subset independent dysbalance of p-Syk/p-Akt in patients with SLE (**Figure 4-14C and Figure 4-6**).

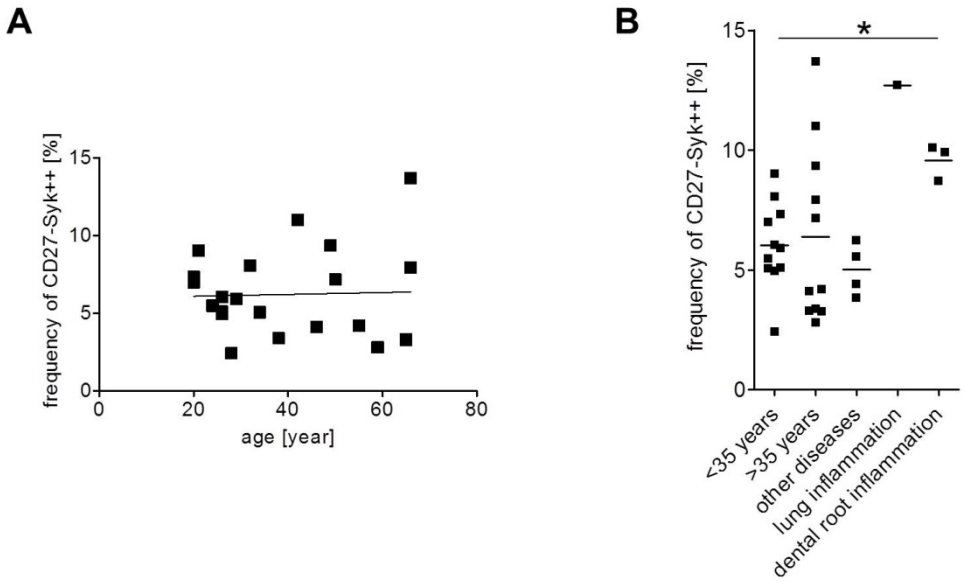

Figure 4-15: Occurrence of CD27-Syk++ B cells in HD is independent of their age but is significantly enhanced in HD suffering from dental root inflammations. A, Spearman's correlation analysis of the frequency of CD27-Syk++ B cells with age (n=22). **B,** Frequency of CD27-Syk++ B cells was analyzed in HD below the age of 35 (n=11), above the age of 35 (n=11), with other diseases including gastro-intestinal disease, sinusitis, gum bleeding/infection (n=4), lung inflammation (n=1) and dental root inflammation (n=3). Each symbol represents an individual subject; horizontal lines show the mean; *p<0.05 by Mann Whitney U test.

Further studies analyzed the occurrence of CD27-Syk++ B cells in HD and revealed no correlation between the frequency of these cells and their age (**Figure 4-15A**). Interestingly, analyzing the frequency of CD27-Syk++ B cells more in detail within HD, a significant enhanced frequency was found in HD suffering from dental root inflammation compared to HD below 35 years (**Figure 4-15B**). Here, HD below the age of 35 had a frequency of 6.0±1.8% compared to 9.6±0.8% in HD with dental root infections (mean±SD, *p<0.05).

Additionally, comparing the frequencies of CD27-Syk++ B cells between HD below and over 35 as well as other inflammatory diseases like gastro-intestinal disease, sinusitis or gum bleeding/infection showed no significant differences (<35 years: 6.0±1.8%, >35 years: 6.4±3.7%, other diseases: 5.0±1.1%). Interestingly, one HD with lung inflammation (pneumonia) showed a frequency of 12.7%.

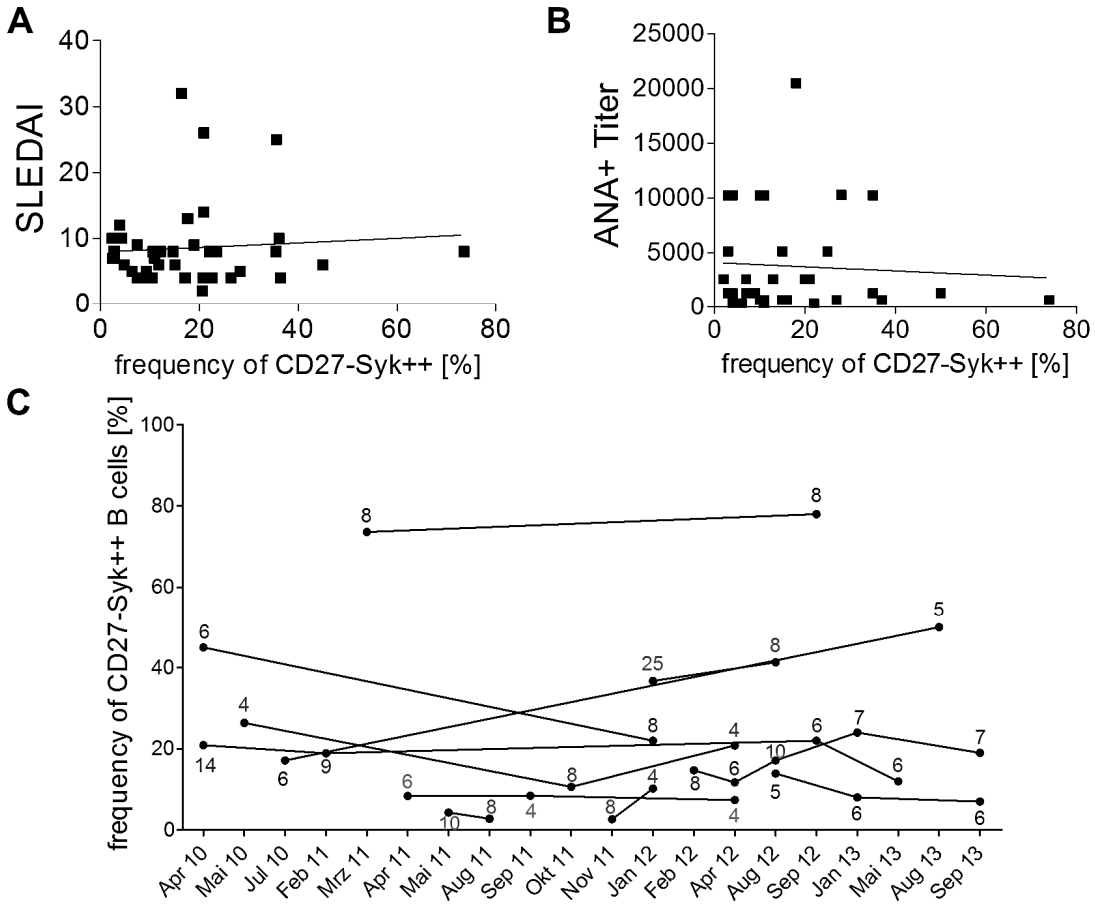

Figure 4-16: Occurrence of CD27-Syk++ B cells in SLE patients is independent of the SLE disease activity index (SLEDAI) or antinuclear antibodies (ANA) and stays stable over time. Spearman's correlation analysis of the frequency of CD27-Syk++ B cells with **(A)** the SLEDAI (n=41) and **(B)** the ANA titer (n=33) did not indicate a functional relationship. **C,** The frequency of CD27-Syk++ B cells were analyzed in 11 SLE patients over a time period of maximal two years showing no changes over time in individual patients. The different numbers show the SLEDAI on the time point analyzed. Each color represents one SLE patient.

Of note, no correlation between the SLEDAI or ANA titer has been found. Furthermore, analyzing the frequency of CD27-Syk++ B cells over time during various follow-ups of several SLE patients remained stable (**Figure 4-16**). This data indicate, that CD27-Syk++ B cells are disease specific and not essentially related to lupus activity.

In addition, whole blood analysis after secondary tetanus/diphtheria (adjuvant alum) vaccinations showed no increased frequency of CD27-Syk++ B cells after 7 or 14 days post vaccination even though the typical increase in plasmablasts/plasma cells was detected indicating that this population is not inducible by tetanus specific *in vivo* immune activation (**Figure 4-17**).

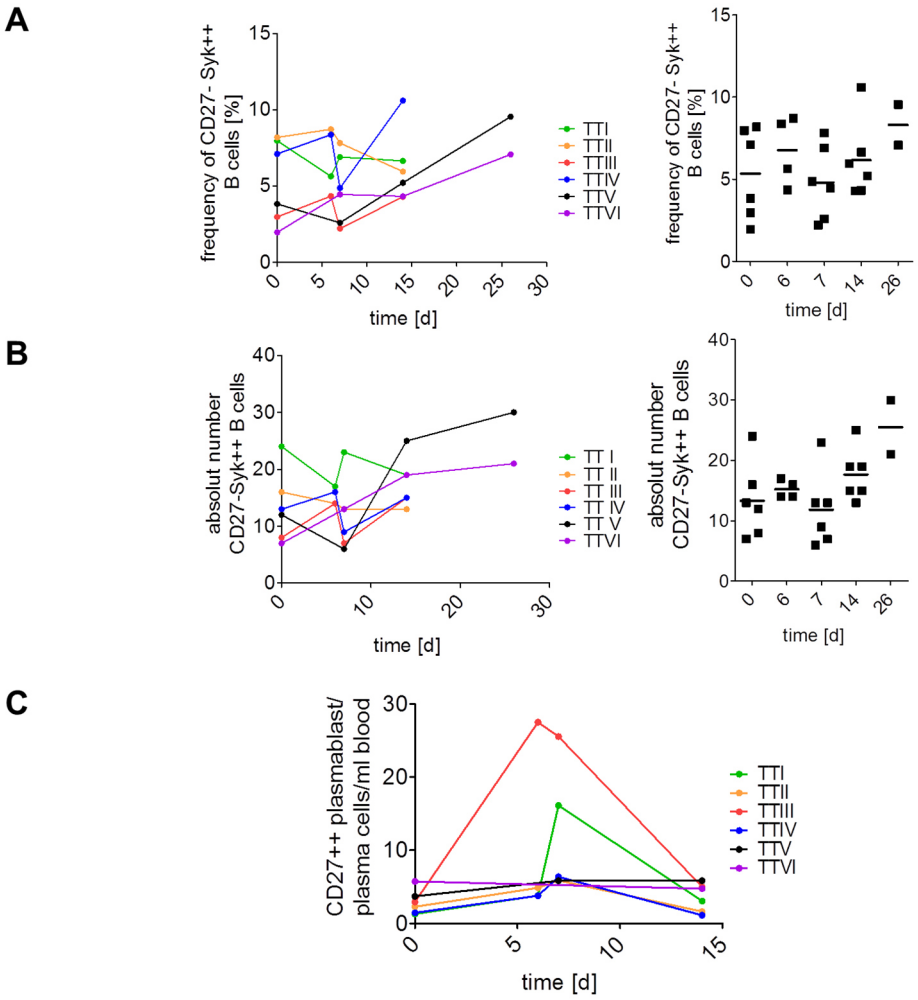

Figure 4-17: Constant frequency of CD27-Syk++ B cells after secondary vaccination with the tetanus/diphtheria toxoid (TT) after day 6, 7 and 14. Whole blood of 6 HD (TTI-TTVI) was analyzed at day 0, 6, 7 and 14 after secondary vaccination with the TT to assess the frequency of CD27Syk++ B cells. Time kinetic and scatter plot show the frequency **(A)** and the absolute numbers **(B)** of CD27-Syk++ B cells after vaccination. Each color or symbol represents an individual subject; horizontal lines show the mean; Wilcoxon's signed rank test. **C,** The absolute number of CD27++ plasmablasts/plasma cells per ml whole blood is shown. The occurrence of CD27-Syk++ B cells is not inducible by *in vivo* immune activation even though the typical increase in plasmablasts/plasma cells was detected after day 6/7 especially in TTI and TTIII.

In accordance with the findings of the tetanus vaccination study, no correlation between the frequency of CD27-Syk++ B cells and the occurrence of CD80+ and CD86+ B cells was found (**Figure 4-18**).

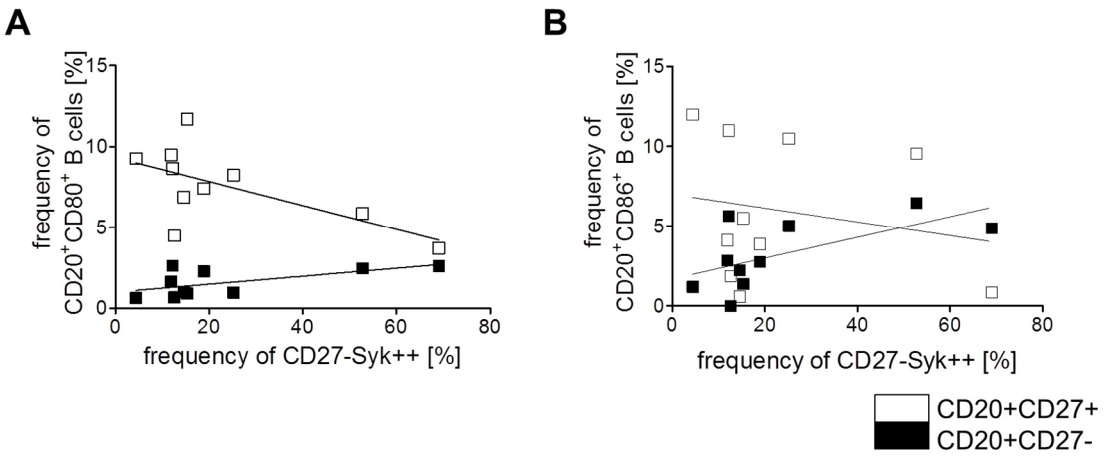

Figure 4-18: The appearance of CD27-Syk++ B cells is independent of a recent *in vivo* immune activation represented by the occurrence of CD80+ and CD86+ B cells. Pearson's correlation between the frequency of CD27-Syk++ B cells and the frequency of CD80+ **(A)** and CD86+ **(B)** within CD27+ (open square) and CD27- (filled square) B cells showed no correlation. Each symbol represents an individual subject of 10 SLE patients.

4.2.2 CD27-Syk++B cells express a distinct phenotype and lack CD38

Several studies described the presence of certain CD27- B cell memory subsets in patients with SLE, such as CD27-IgD-, CD27-IgD-CD95+ or $CD21^{low}$ B cells (36, 138, 185). Therefore, a detailed phenotypic study of CD27-Syk++ B cells was performed to analyze if this subset might share specific characteristics with one of the already known subsets mentioned above.

Interestingly, careful studies of CD27-IgD-, CD27-IgD-CD95+ and CD27-CD21- B cells revealed that not all subsets express homogenously Syk++. Here, only 39.2±24.7% of the CD27-IgD-, 67.4±22.8% of the CD27-IgD-CD95+ and 64.2±20.9% of the CD27-CD21- B cell subsets were found to carry Syk++ (median±SD, **Figure 4-19A**).

By contrast, 31.9±11.6% of the CD27-Syk++ B cell subset were neither CD27-IgD-, CD27-IgD-CD95+ nor CD27-CD21-. Thus, the CD27-Syk++ B cell subsets represents a unique B cell subset enhanced in SLE patients and has not been fully described by previous studies (**Figure 4-19B**).

In addition, CD27-Syk++ B cells were found to uniquely lack the expression of the type II transmembrane glycoprotein CD38 (**Figure 4-19C**). CD38 expression is tightly regulated during B cell development and downregulated during the late B cell memory phase (186, 187). CD38 has been reported to play an important role as BCR co-receptor modulating its threshold (186, 188). However, the exact function of CD38 appears contradictory and not well characterized (187).

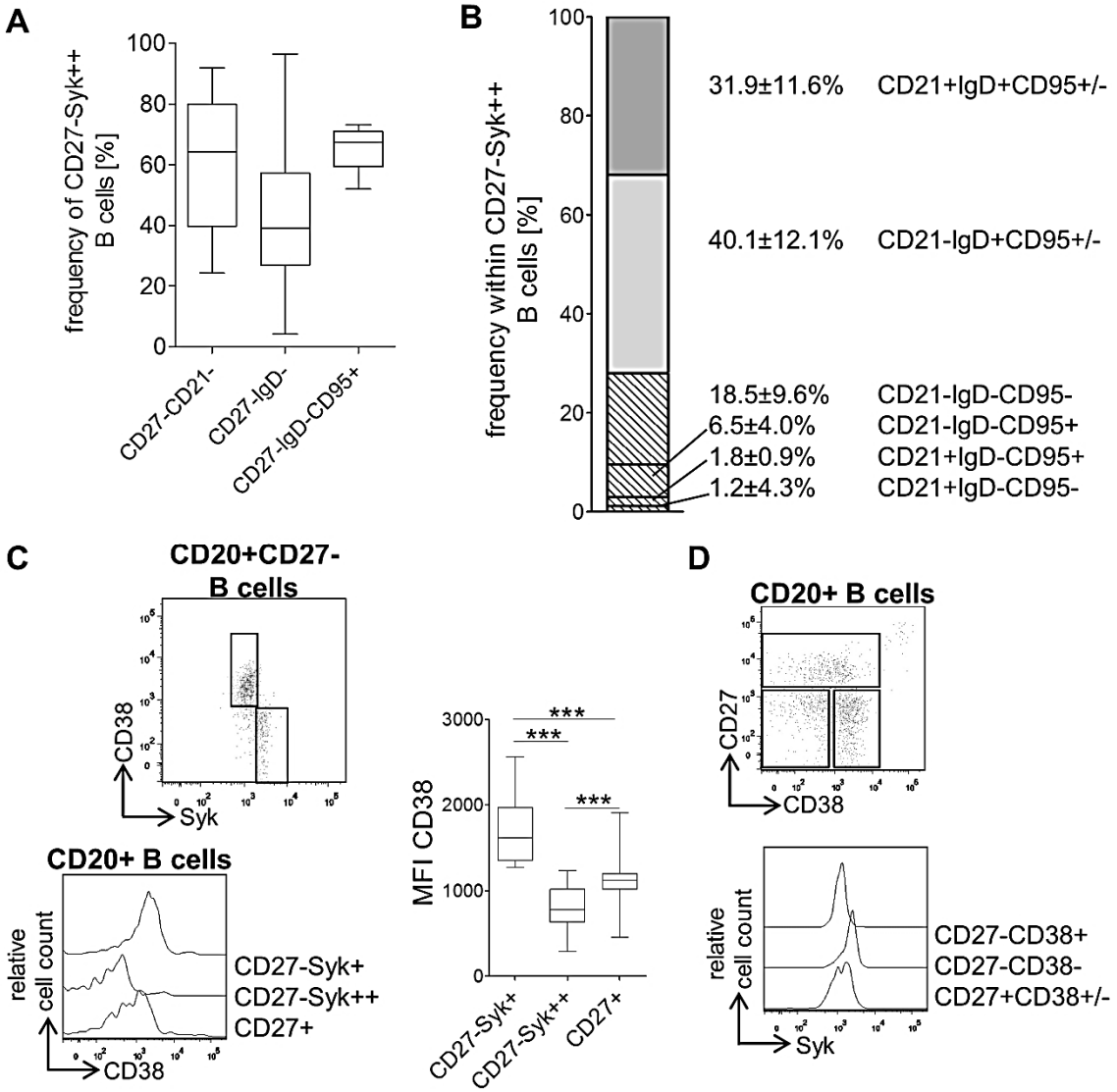

Figure 4-19: CD27-Syk+ B cells from SLE patients lack the expression of CD38 and cover only partially previously described B cell abnormalities. A, Box and whisker plots show the frequency of Syk++ cells within the CD27-CD21-, CD27-IgD-, and CD27-IgD-CD95+ B cell subsets (n=5–16 samples). **B,** Composition of CD27-Syk++ B cells according to their expression of CD21, IgD, and CD95. Values are the mean±SD of 18 samples. **C,** Representative histograms show the expression of CD38 on CD27-Syk+, CD27-Syk++, and CD27+ B cells. Dot plot demonstrates that CD27-Syk++ cells lack CD38 expression. Box and whisker plots show the MFI of CD38 on CD27-Syk+, CD27-Syk++, and CD27+ B cells (n=11 samples). Each box represents the 25% and 75% confidence limits. Lines inside the boxes represent the median. ***p<0.001 by Wilcoxon's signed rank test. **D,** CD27-CD38-, CD27-CD38+, and CD27+CD38+/- B cells were gated and their corresponding Syk expression is shown in a representative histogram.

Analysis of the MFI values of CD38 of CD27-Syk++ B cells showed a significant reduced expression compared to CD27-Syk+ and CD27+ B cells (CD27-Syk++: 777±258, CD27-Syk+: 1620±401, CD27+ 1122±361; median±SD, ***p<0.001). Analyzing the expression of Syk in sorted CD27-CD38- B cells revealed an enhanced Syk expression, confirming that the lack of CD38 as a unique characteristic of CD27-Syk++ B cells (**Figure 4-19D**).

Furthermore, analyzing the baseline level of phosphorylated Syk showed significantly elevated levels of p-Syk(Y352) in CD27-Syk++ compared to CD27-Syk+ and CD27+ B cells reflecting an pre-activated phenotype (CD27-Syk++: 569±211, CD27-Syk+: 291±115, CD27+ 367±125; mean±SD, ***p<0.001; **Figure 4-20A**).

Previously, a comparable cellular distribution of Syk within the cytoplasm and the nucleus in unstimulated B cells has been described (189). In order to identify potential differences of the cellular localization of Syk within CD27-Syk+, CD27-Syk++ and CD27+ B cells, the distribution of Syk together with IgM as membrane and DAPI as nuclear staining were determined in freshly obtained blood from SLE patients using confocal microscopy.

The fluorescence intensity of Syk in the cytoplasm and the nucleus was analyzed by calculating the mean intensity in these two subcellular areas using a cellular histogram. As shown in **Figure 4-20B and C**, CD27-Syk++ cells expressing IgM showed an overall increased Syk intensity in the nucleus as well as in the cytoplasmic region. CD27-Syk++IgM+ cells had a cytoplasmic mean Syk fluorescence intensity of 8666±4501 compared to 3796±1780 in CD27-Syk+IgM+ and 4326±2818 in CD27+IgM+ memory B cells, respectively (mean±SD, ***p<0.001; **Figure 4-20B**).

In the nucleus, the mean Syk fluorescence intensity was 6498±2957 for CD27-Syk++IgM+ cells compared to 3386±950 for CD27-Syk+IgM+ and 4299±2134 within CD27+IgM+ B cells, respectively (mean±SD; **Figure 4-20B, C**). Further, CD27-Syk++IgM+ B cells showed a significantly higher Syk fluorescence intensity in the cytoplasm region compared to the nucleus, which has neither been observed for CD27-Syk+IgM+ nor for CD27+IgM+ memory B cells (***p<0.001).

Taken together, the accumulation of Syk at its functional site (membrane IgM and cytoplasm) in CD27-Syk++ B cells differs substantially to the classical naïve and memory B cells and confirms the pre-activated or recently activated state of these B cell subset.

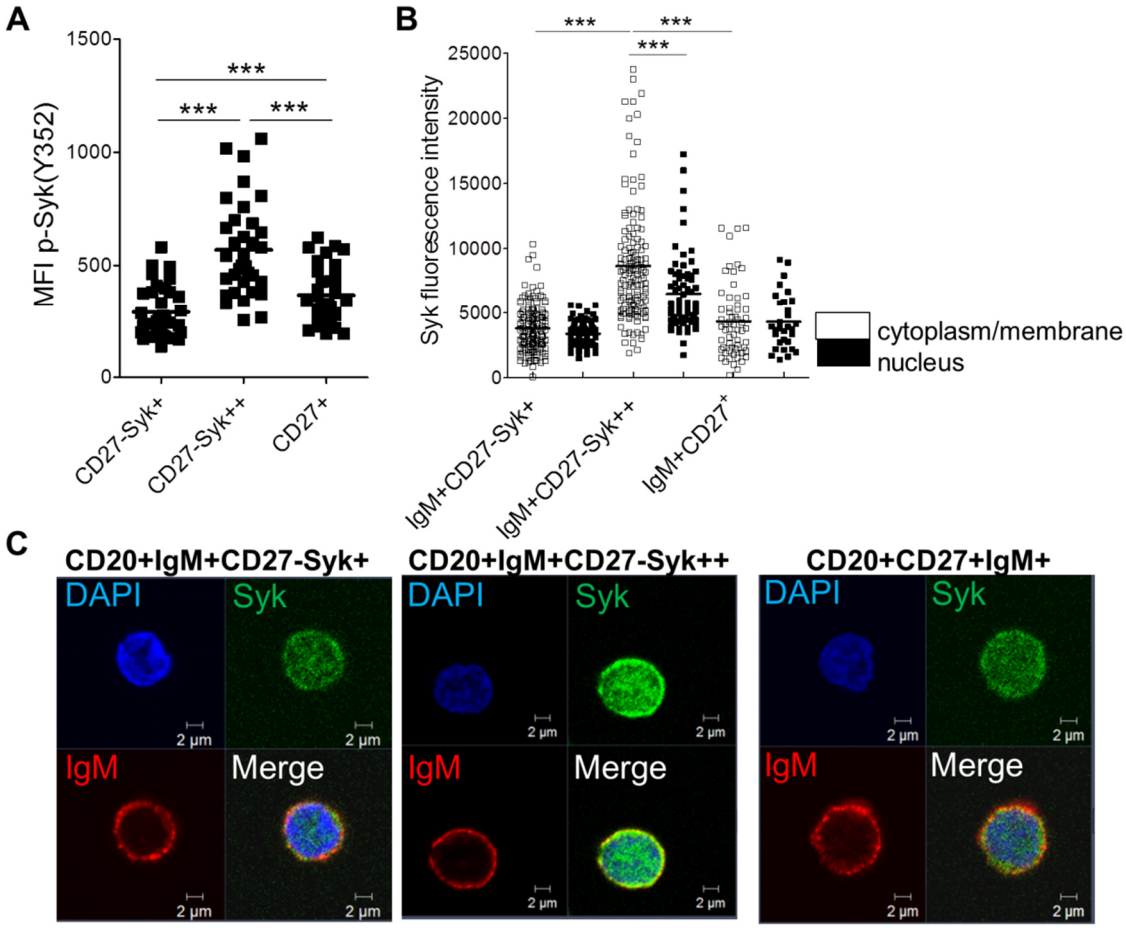

Figure 4-20: CD27-Syk++ B cells from SLE patients showed enhanced expression of basal p-Syk(Y352) and a preferential accumulation of Syk in the cytoplasm compared to CD27-Syk+ and CD27+ B cells. A, Whole blood analysis of CD27-Syk+, CD27-Syk++, and CD27+ B cells from SLE patients (n=34) according to their basal expression of p-Syk(Y352) showed enhanced expression (MFI values) of p-Syk(Y352) in CD27-Syk++ B cells. Each symbol represents an individual subject; horizontal lines show the mean; ***p<0.001 by Wilcoxon's signed rank test. **B** and **C,** Fixed and permeabilized sorted B cells were stained against IgM (red) and analyzed by confocal microscopy. DAPI (blue) was used to stain the nucleus (original magnification: 630x). The Syk fluorescence intensity within CD27-Syk+IgM+, CD27-Syk++IgM+ and CD27+IgM+ B cells was determined by cellular histograms (DAPI (blue), Syk-FITC (green) and IgM-RRX (red)) across an individual cell and analyzed according to their membrane/cytoplasmic (DAPI-; filled square) and nuclear (DAPI+; open square) Syk expression. Each symbol represents an individual B cell of 3 SLE patients; horizontal lines show the mean; ***p<0.001 by Wilcoxon's signed rank test (≥15 cells/B cell subset).

Beyond the lack of CD38, enhanced Syk and p-Syk expression and an accumulation of Syk in the cytoplasm compared to the nucleus, a significantly higher expression of the B cell lineage markers CD19 and CD20 by CD27-Syk++ B cells were observed compared to CD27-Syk+ as well as CD27+ memory B cells (CD27-Syk++: CD19 1539±398, CD20 1347±1571; CD27-Syk+: CD19 1138±189, CD20 1228±1140; CD27+: CD19 1282±265, CD20 1160±1137; median±SD, **p<0.01, ***p<0.001; **Figure 4-21A, B**).

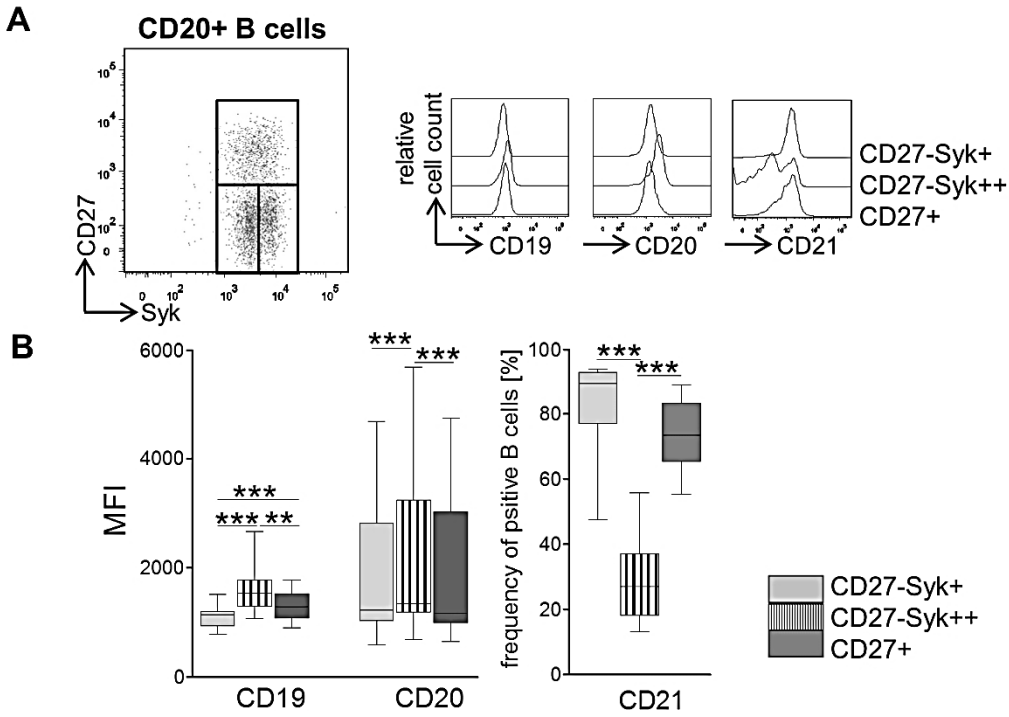

Figure 4-21: CD27-Syk++ B cells show a unique phenotype. A, Gating strategy of CD27-Syk+, CD27-Syk++, and CD27+ B cells (left). Representative histograms are shown (right) for the expression of CD19, CD20 and CD21. **B,** MFI of CD19 (n =17) and CD20 (n =29) and the frequency of CD21+ (n=14) of CD27-Syk+ (light grey), CD27-Syk++ (strips), and CD27+ (dark grey) B cells are shown. Data are represented as box and whisker plots. Each box represents the 25% and 75% confidence limits. Lines inside the boxes represent the median. Lines outside the boxes represent the minimum and maximum values. **p<0.01, ***p<0.001 by Wilcoxon's signed rank test.

In addition, comparing the expression of the C2 complement receptor (CD21) showed a significantly lower percentage of CD21 expressing B cells within CD27-Syk++ (27.0±13.3%) compared to CD27-Syk+ (89.3±13.6%) or CD27+ (73.5±10.3%) B cells, respectively (median±SD, ***p<0.001; **Figure 4-21A, B**).

To characterize the phenotype of CD27-Syk++ B cells in further detail, the Ig heavy chain isotype expression by CD27-Syk+, CD27-Syk++ and CD27+ B cells was examined. An increase of pre-switch IgM+ only (CD27-Syk+ 7.9±6.3%, CD27-Syk++ 14.4±13.1%) and post-switch IgM-IgD- memory B cells (CD27-Syk+ 6.7±8.5%, CD27-Syk++ 25.8±21.5%) as well as a decrease of IgM+IgD+ B cells (CD27-Syk+ 78.4±12.5%, CD27-Syk++ 51.0±18.5%) was observed among CD27-Syk++ compared to CD27-Syk+ B cells (mean±SD; **Figure 4-22A, B**). However, comparing the frequency of IgM+ only, IgM-IgD- and IgM+IgD+ B cells showed a distinct distribution between CD27-Syk++ and classical CD27+ memory B cells (CD27+: IgM+ 39.5±16.9%, IgM-IgD- 47.9±17.8%, IgM+IgD+ 10.3±6.4%). Therefore, CD27-Syk++ B cells contain an increased frequency of IgM+ only and IgM-IgD- post-switch memory B cells compared to CD27-Syk+ B cells but decreased frequency compared to conventional CD27+ memory B cells.

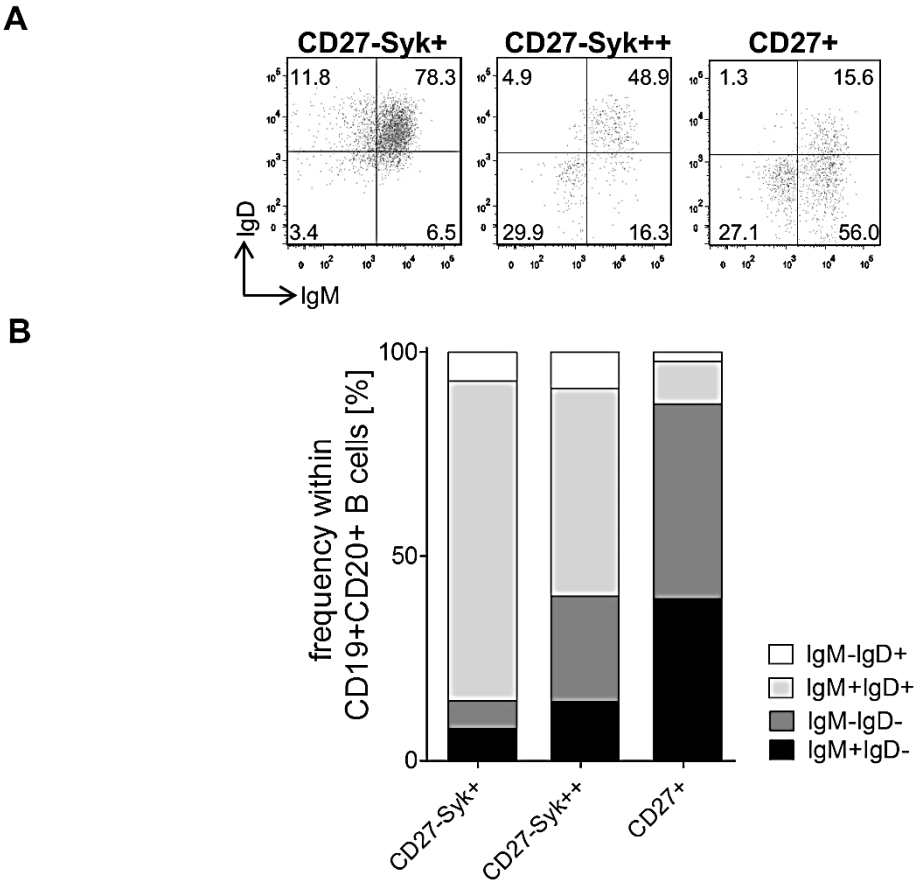

Figure 4-22: CD27-Syk++ B cells contained a distinct frequency of IgM+ only and IgM-IgD- post-switch memory B cells. A, Representative dot plots are shown for the expression of IgM and IgD by CD27-Syk+, CD27-Syk++ and CD27+ B cells. Gating strategy of four different B cell subsets regarding their IgM and IgD expression. **B,** Stacked bars represent the mean frequency of "IgM+ only" pre-switch memory (black), IgM+IgD+ naïve (light grey), IgD+ only naïve (white) and IgM-IgD- post-switch memory B cells (dark grey) within CD27-Syk+, CD27-Syk++ and CD27+. Data are represented as mean from 18 independent experiments.

Taking together, the CD27-Syk++ B cells does not completely overlap with the previously recorded CD27- memory B cell subsets described in SLE patients. These cells lack the expression of CD38, show enhanced levels of p-Syk(Y352) and an accumulation of Syk in the cytoplasm as well as an enhanced expression of CD19 and CD20. In addition, CD27-Syk++ B cells showed reduced frequencies of CD21+ cells and a divergent frequency of IgM+ only and IgM-IgD- post-switched memory B cells.

4.2.3 CD27-Syk++B cells share memory B cell characteristics

Although CD27 can serve as a reliable surface marker for memory B cells (27), CD27-IgD-CD95+ memory-like B cells correlating with SLE activity (36), low mutated and isotype-switched CD27-IgD- memory B cells with an increased frequency in SLE patients, human immunodeficiency virus (HIV) infections and elderly people have been identified

(32, 33, 36, 37, 185). Thus, CD27 has apparently limitations as marker for memory B cells in certain immune diseases.

Therefore, other additional markers are needed to identify memory B cells, such as SHM of V(D)J gene rearrangements, Ig class-switching (23, 24, 190) as well as an inactive ABC-B1 transporter (38). In this regard, analysis of the ABC-B1 transporter activity was measured by the export of the MTO dye. As expected and previously reported (38) CD27+ memory B cells failed to exclude the dye and thus remained MTO+ (92.1±5.3%). CD27-Syk+ naïve B cells expressed an active ABC-B1 transporter and were MTO- (13.7±13.5%). Interestingly, CD27-Syk++ B cells showed a similar ABC-B1 transporter activity compared to CD27+ memory B cells (MTO+ 86.8±7.5%) consistent with the conclusion that these cells do not represent a conventional naïve B cell subset and represent rather memory-like B cells (median±SD, *$p<0.05$, ***$p<0.001$; **Figure 4-23A, B**).

Furthermore, the frequency of CD95+ B cells, was significant increased in the CD27-Syk++ (22.4±14.6%) compartment compared to CD27-Syk+ cells (1.8±1.9%) but similar to CD27+ B cells (25.5±12.0%; median±SD, ***$p<0.001$; **Figure 4-23A, B**). Neither one of the three B cell subsets analyzed contained substantial numbers of Ki67+ B cells, which indicates that >99% of CD27-Syk++ cells had not recently proliferated (CD27-Syk++: 0.94±0.44%, CD27-Syk+: 0.59±0.67%, CD27+ 1.10±3.24; median±SD, **$p<0.01$; **Figure 4-23A, B**).

Additionally, analysis of the mutation frequency of CD27-Syk++ and CD27-CD38-B cells showed an average mutation frequency of 7.2±5.9% (mean±SD; **Figure 4-23C**) which is consistent with reported mutation frequencies of 4-12% in peripheral memory B cells (191), whereas CD27-CD38+ naïve B cells did not express somatic hypermutations within V(D)J regions (mutation frequency 0%). Interestingly, also three sorted CD27-Syk++(CD38-) clones did not show any mutations within the heavy chain.

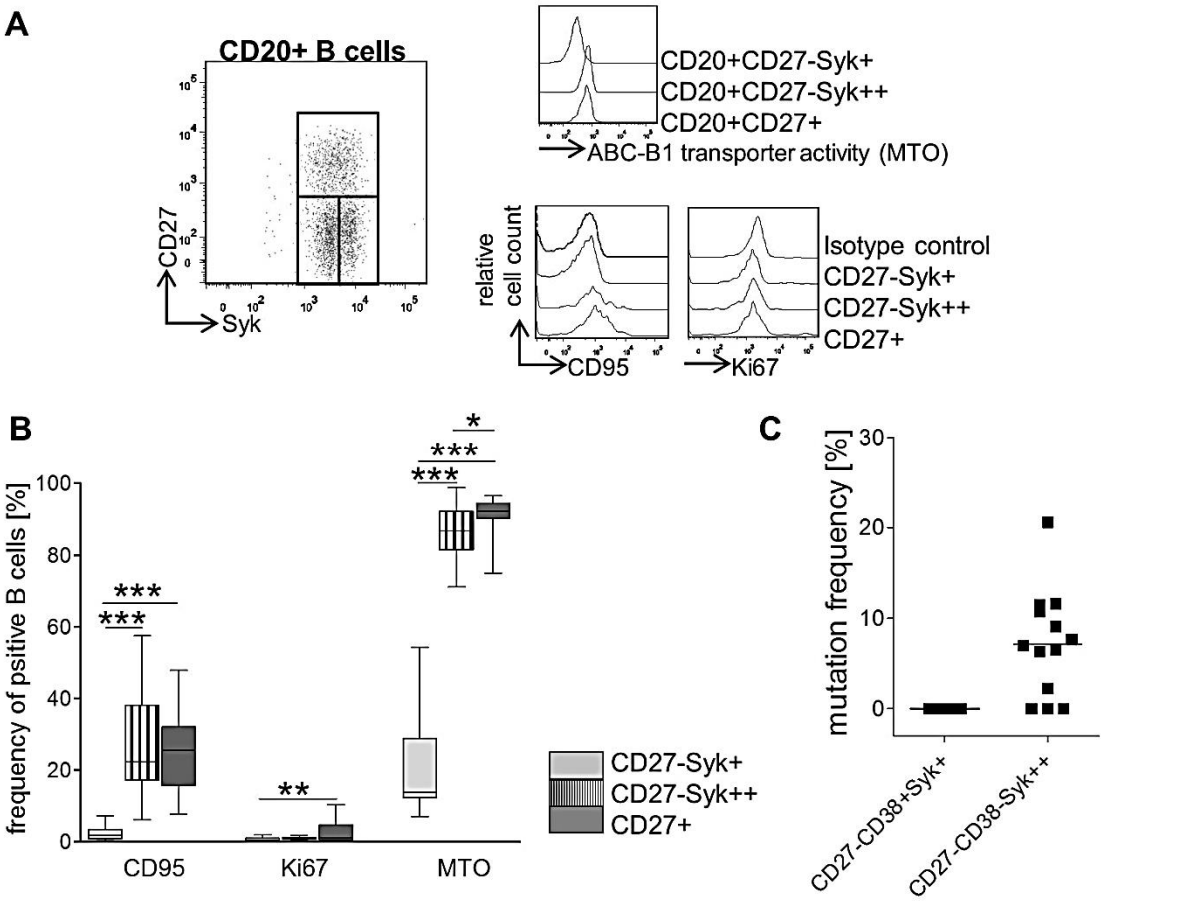

Figure 4-23: CD27-Syk++ B cells showed a memory-like phenotype. A, Gating strategy of CD27-Syk+, CD27-Syk++, and CD27+ B cells (left). Representative histograms are shown (right) for the expression of CD95, and Ki-67, as well as the activity of the ABC-B1 transporter by MitoTracker Orange [MTO] exclusion. **B,** Frequency of CD95+ (n=16), Ki-67+ (n= 11), and MTO+ (n=19) cells in CD27-Syk+, CD27-Syk++, and CD27+ B cells are shown. Data are represented as box and whisker plots. Each box represents the 25% and 75% confidence limits. Lines inside the boxes represent the median. Lines outside the boxes represent the minimum and maximum values. *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon's signed rank test. **C,** Sorted single CD27-Syk+(CD38+) and CD27-Syk++(CD38-) B cells were analyzed regarding their mutation frequency of B cell receptor gene rearrangements. Included are 5 IgM sequences from CD27-CD38+ B cells and 5 IgM, 1 IgG, and 7 IgA sequences from CD27-Syk++ and CD27-CD38- B cells. Intracellular staining's were performed for SLE patients no. 1–3. CD27-Syk+ and CD27-Syk++ B cells were sorted and further analyzed for their V_HDJ_H gene rearrangements. Surface staining was performed for cells from SLE patient no. 4. Subsequently, CD27-CD38+ and CD27-CD38- B cells were sorted and analyzed. Each symbol represents an individual sample; horizontal lines show the mean.

Additionally, analysis of the mutation frequency of CD27-Syk++ and CD27-CD38- B cells showed an average mutation frequency of 7.2±5.9% (mean±SD; **Figure 4-23C**) which is consistent with reported mutation frequencies of 4-12% in peripheral memory B cells (191), whereas CD27-CD38+ naïve B cells did not express somatic hypermutations within V(D)J regions (mutation frequency 0%). Interestingly, also three sorted CD27-Syk++(CD38-) clones did not show any mutations within the heavy chain.

The overall data are consistent with the conclusion that CD27-Syk++ B cells clearly differ in their phenotype from conventional CD27-Syk+ naïve cells and express phenotypic and especially functional characteristics more typical of memory B cells

lacking the memory marker CD27. Thus, these cells are a unique CD27- subset that is enhanced in SLE patients and reflects a pre-activated memory-like B cell subset.

4.2.4 Enhanced differentiation of CD27-CD38- into CD27++ and Ab secreting B cells

Subsequent experiments thought to determine whether CD27-Syk++ B cells show functional differences compared to classical naïve CD27-Syk+ B cells. Since the discrimination of CD27-Syk+ with CD27-Syk++ B cells required fixation and permeabilization, CD27-CD38- B cells that were largely Syk++ were used for functional analysis (**Figure 4-19C**). Three B cell subsets regarding their CD27 and CD38 expression were sorted: the Syk expression prior to the experiments of CD27-CD38- and CD27-CD38+ B cells were verified as shown in **Figure 4-19D.** CD27-CD38- B cells showed an enhanced differentiation into CD27+ and CD27+CD138++ cells in contrast to CD27-CD38+ B cells after 5 days of *in vitro* stimulation (**Figure 4-24A**).

In detail, CD27-CD38+ B cells stimulated with IL-2, IL-10, CpG and anti-IgM/IgG differentiated into CD27++ B cells at a frequency of 6.2±4.9% and only 0.5±0.4% were CD27++CD138++, whereas 20.0±11.8% CD27++ and 1.7±1.3% CD27++CD138++ cells were found for CD27-CD38- B cells. Comparable levels of CD27++ and CD27++CD138++ B cells were observed in the CD27+CD38+/- B cell memory subset (CD27+CD38+/-: 62.5±13.3% CD27++ and 4.1±2.8% CD27++CD138++; mean±SD; **Figure 4-24B**).

These results were consistent with the concentration of IgG measured by ELISA in culture supernatants and the number of Ab secreting cells (ASCs) in the corresponding cultures (**Figure 4-25**): CD27-CD38- cells representing CD27-Syk++ cells produced substantially higher IgG *in vitro* compared to CD27-CD38+ cells (**Figure 4-25A**). Moreover, the secretion of IgG by the different B cell subsets was confirmed by ELISPOT (**Figure 4-25B**) showing increased differentiation into IgG secreting cells by CD27-CD38- B cells compared to CD27-CD38+ B cells.

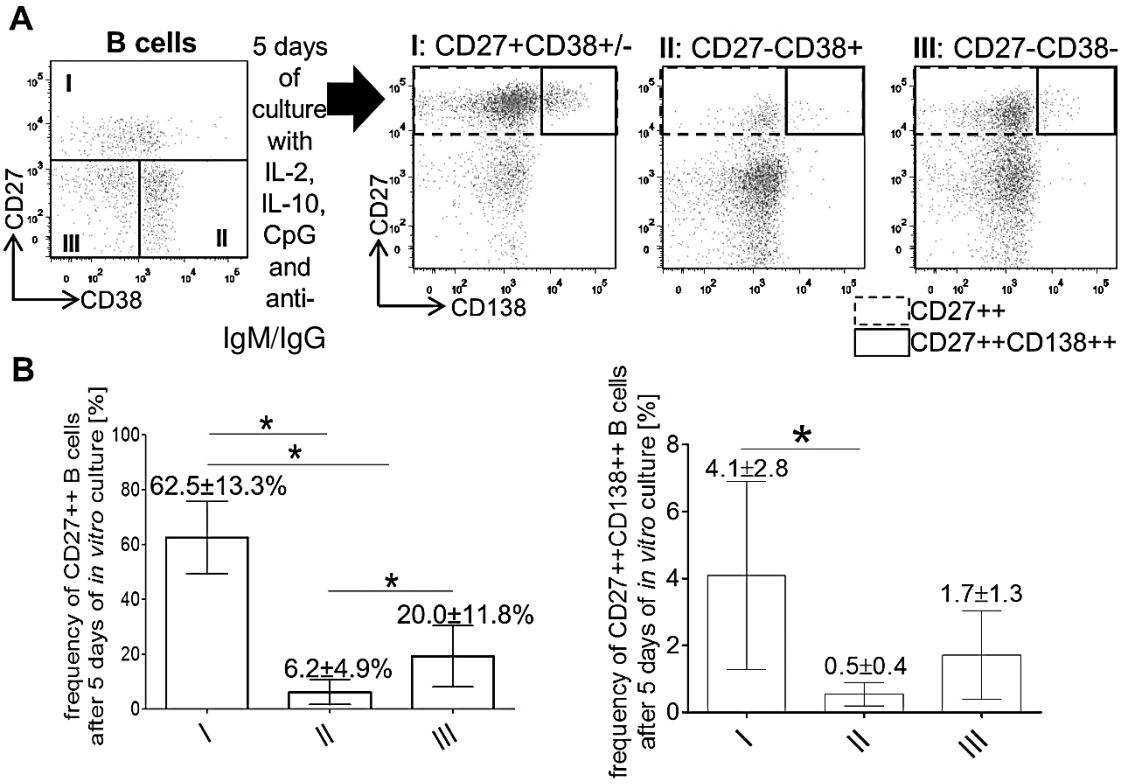

Figure 4-24: Enhanced differentiation of CD27-Syk++ into CD27++ B cells compared to CD27-Syk+ cells after 5 days *in vitro* stimulation. A, Gating strategy of CD19+CD20+ B cells for sorting CD27+CD38+/- (gate I), CD27-CD38+ (gate II), and CD27-CD38- (gate III) B cell subsets based on the expression of CD27 and CD38. Dot plots show the expression of CD27 and CD138 on CD27+CD38+/- (gate I), CD27-CD38+ (gate II), and CD27-CD38- (gate III) B cells after 5 days *in vitro* culture with IL-2, IL-10, CpG, and anti-IgM/IgG. **B,** The frequencies of CD27++ and CD27++CD138++ B cells were determined in the 3 gated subsets showing enhanced frequencies of CD27++ and CD27++CD138++ B cells in CD27-CD38- (gate III) compared to CD27-CD38+ (gate II) B cells. Values are represented as mean±SD of 6 independent experiments; *p<0.05 by Wilcoxon's signed rank test.

The differentiation of self-reactive B cells into auto-ASCs plays a crucial role in the pathogenesis of SLE. Therefore, the differentiation capacity of CD27-CD38- B cells into auto-ASCs was analyzed by testing supernatants for the presence of auto-Ab using the human epithelial type 2 (Hep-2) cell system as substrate coated on microscopy slides for human auto-Ab. Incubating the supernatants on the slides coated with Hep-2 cells, no autoreactive IgG obtained from HD was detected, whereas all three SLE patients showed auto-Ab production against cellular antigens (mainly cytoplasmic reactivity) from CD27+ memory B cells as shown in **Figure 4-25C.**

Of note, ASCs derived from CD27-CD38- cells from one out of three SLE patients showed staining of Hep-2 cells against cytoplasmic autoantigens. Although IgG has been produced by ASCs derived from normal CD27-CD38+ as shown by ELISA and ELISPOT, auto-Abs were not detectable in this assay. Together, CD27-CD38-(Syk++) showed an enhanced differentiation capacity into IgG secreting PC compared to the CD27-CD38+(Syk+) B cell subset.

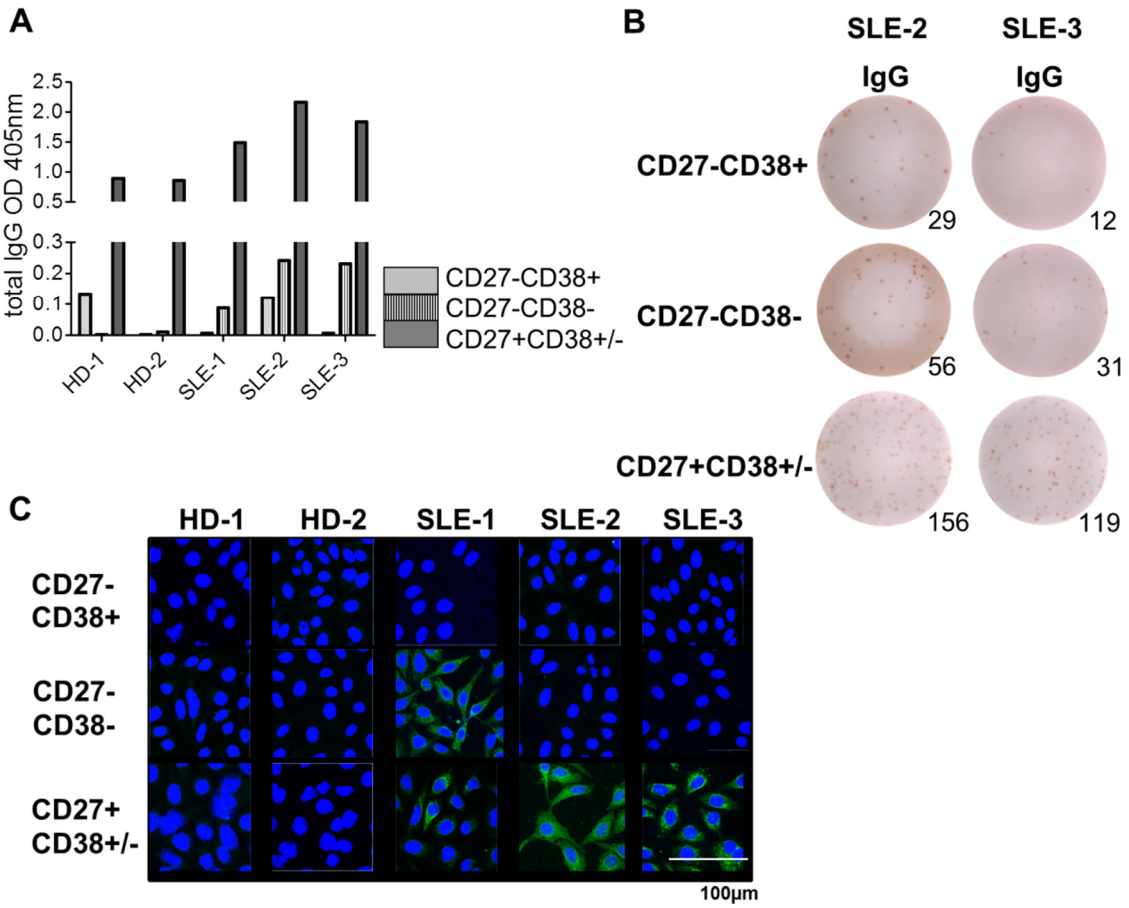

Figure 4-25: CD27-Syk++ B cells differentiated after 5 days *in vitro* stimulation into IgG secreting (auto)-Ab producing B cells. A, Total IgG secreted into supernatants of CD27+CD38+/-, CD27-CD38+, and CD27-CD38- B cells stimulated with IL-2, IL-10, CpG, and anti-IgM/IgG after 5 days of *in vitro* culture from 2 HD and 3 SLE patients. **B,** Enzyme-linked immunospot analyses of $0.5x10^5$ sorted CD27+CD38+/-, CD27-CD38+, and CD27-CD38- B cells from 2 SLE patients. Cells were stimulated *in vitro* for 5 days with IL-2, IL-10, CpG, and anti-IgM/IgG. Spots representing IgG Ab-secreting cells were counted. Numbers indicate spots of Ab-forming cells. Representative examples are shown. **C,** Autoreactive IgG in supernatants from the 5 days B cell cultures from CD27+CD38+/-, CD27-CD38+, and CD27-CD38- B cells subsets derived from the 2 HD and from the 3 SLE patients shown in **A**. Autoreactive IgG binding to cytoplasmic antigens was determined by confocal microscopy using HEp-2 cells; original magnification x630.

4.2.5 Stimulation with IFN-γ, LPS or TNF-α leads to increased frequencies of CD27-Syk++ B cells

To analyze the nature of CD27-Syk++ B cells, whole blood was stimulated with different agents that have been previously described to be involved either in autoimmune diseases, such as IFN-γ (125), TNF-α, IL-6 (124), and BAFF (192) or abnormal B cell responses, such as LPS and CpG (193).

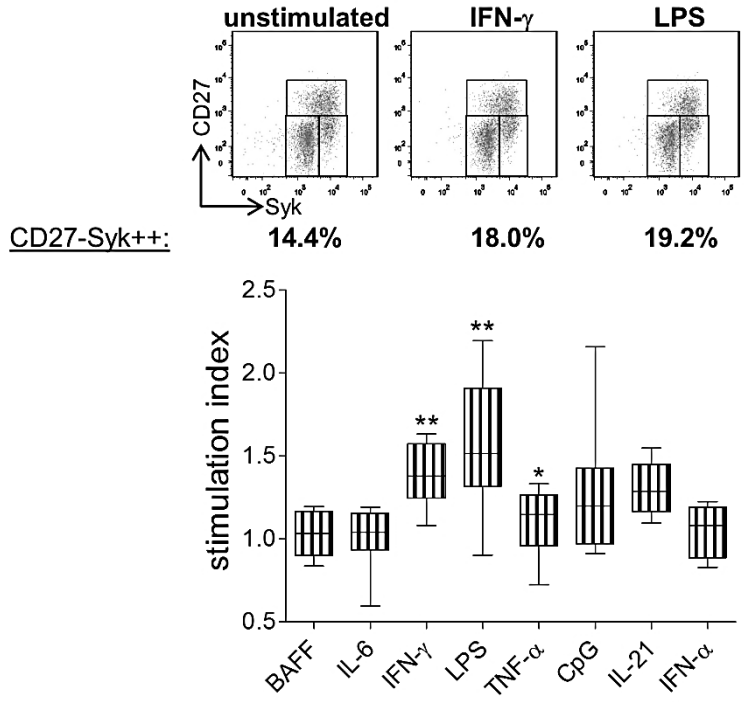

Figure 4-26: Stimulation with IFN-γ, LPS, or TNF-α induced the generation of CD27-Syk++ B cells *in vitro*. A, Median frequencies of CD27-Syk++ B cells after 2 days of stimulation with IFN-γ, TNF-α or LPS, as compared to unstimulated whole blood. Representative dot plots are shown. **B,** Stimulation index (frequency of CD27-Syk++ cells among total CD20+ B cells stimulated/unstimulated) after 2 days of *in vitro* culture with either BAFF, IL-6, IFN-γ, LPS, TNF-α, CpG, IL-21, or IFN-α as compared to unstimulated cells (n=12 for each analysis except IL-21 (n =8) and IFN-α (n=4)). Data are shown as box and whisker plots. Each box represents the 25% and 75% confidence limits. Lines inside the boxes represent the median. Lines outside the boxes represent the minimum and maximum values. *p<0.05, **p<0.01 by Wilcoxon's signed rank test.

After 48 h *in vitro* stimulation with the typical Th1-cytokines (41) IFN-γ, TNF-α or LPS a substantial increase of CD27-Syk++ B cells was observed (**Figure 4-26**).

Here the stimulation index (frequency of CD27-Syk++ stimulated/unstimulated) increased substantially to 1.4±0.2 after IFN-γ stimulation (**p<0.01), to 1.2±0.2 after TNF-α treatment (*p<0.05) and to 1.5±0.4% after LPS stimulation (**p<0.01). Stimulation with BAFF (1.0±0.2), IL-6 (1.0±0.2), CpG (1.2±0.4), IL-21 (1.3±0.2) or IFN-α (1.1±0.2) showed no significant changes in the frequency of CD27-Syk++ B cells (median±SD; **Figure 4-26**).

In summary, this data indicate, that increased availability of certain cytokines in patients with SLE especially the Th1 specific cytokines INF-γ and TNF-α in contrast to the typical Th2 cytokine IL-6 may be able to induce the generation of CD27-Syk++ B cells. In line with this, secondary vaccination studies with tetanus did not induce the generation of CD27-Syk++ B cells *in vivo*.

4.2.6 sCD27 does not correlate with the occurrence of CD27-Syk++ B cells

The co-stimulatory molecule CD27 can be released or shed from the cell surface by metalloproteinases after lymphocyte activation (194). Increased levels of soluble CD27 (sCD27) have been found in anti-CD3 stimulated T cells *in vitro* (195). In addition, sCD27 has been found in serum, plasma and urine samples in HD but was especially increased in patients with SLE indicating that sCD27 reflects an increased immune activation *in vivo* (194). Therefore, to check if CD27-Syk++ B cells are generated by the shedding of CD27, the concentration of sCD27 was compared with the frequency of CD27-Syk++ B cells in the serum of SLE patients as well as in the supernatant of 2 days *in vitro* stimulated whole blood B cell cultures. Analyzing the soluble form of CD27 by an ELISA assay showed no correlation between the concentration of sCD27 (U/ml) in the serum and the frequency of CD27-Syk++ B cells in the periphery of SLE patients (**Figure 4-27A**).

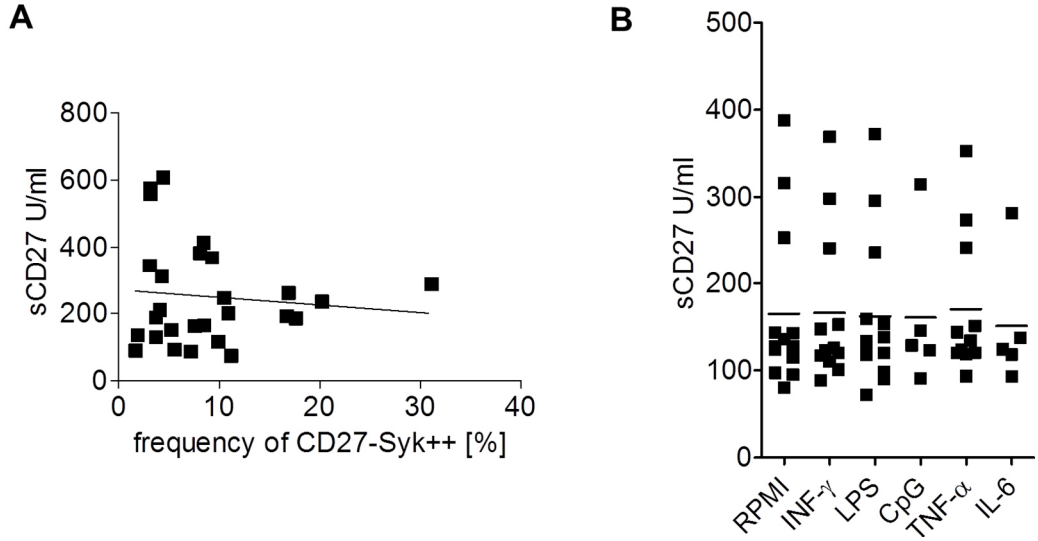

Figure 4-27: The occurrence of CD27-Syk++ B cells in patients with SLE do not correlate with the concentration of sCD27 in the serum or in cell culture supernatants. A, Pearson's correlation analysis of the frequency of CD27-Syk++ B cells with the concentration (U/ml) of soluble CD27 (sCD27) in the serum of 27 SLE patients is shown. **B,** Total sCD27 secreted into supernatants was analyzed from stimulated whole blood *in vitro* cultures after 2 days of stimulation with IL-6, IFN-γ, LPS, TNF-α, CpG or RPMI (control). Each symbol represents an individual sample of 5 SLE patients; horizontal lines show the mean, Wilcoxon's signed rank test.

In addition, no differences between sCD27 has been observed between whole blood B cell culture supernatants unstimulated (RPMI) or stimulated with INF-γ, LPS, CpG, TNF-α or IL-6 even though the stimulation with INF-γ, LPS and TNF-α led to an enhanced frequency of CD27-Syk++ B cells *in vitro* (**Figure 4-27B**).

This observation permits the conclusion, that the lack of CD27 expression on CD27-Syk++ B cells is likely not related to an enhanced shedding of CD27 that would result in enhanced levels of sCD27. However, since B cells represent a minor fraction of

cells within whole blood, the detection sensitivity could be insufficient to detect minor changes of B cell specific sCD27.

4.3 Summary

In summary, the comprehensive analysis of the BCR complex and the downstream signaling cascade in B cells of patients with SLE compared to HD revealed fundamentally and hitherto unknown molecular differences which could play an essential role in the development and maintenance of autoimmunity.

First, the analysis of the expression profile of the BCR co-receptors CD19 and CD22 revealed an enhanced expression and activation of the inhibitory co-receptor CD22 on CD27- B cells of SLE patients. Furthermore, CD27- B cells from SLE patients showed an imbalanced expression of CD19/CD22 which strongly correlated with the ratio of p-Syk/p-Akt after BCR activation in CD27- but also CD27+ B cells. This indicates a striking interrelation between the surface expression of CD22 and CD19 and the balance of the p-Syk/p-Akt signaling pathways.

In addition, SLE patients in comparison to HD showed a hyporesponsive BCR reactivity regarding the phosphorylation of Syk, PLC-γ2 and, finally intracellular Ca^{2+} release upon anti-IgM/IgG stimulation which was independent of the BCR isotype. Simultaneously, hyperactive Akt activation was observed in patients with SLE leading to a shifted balance of p-Syk/p-Akt towards the pro-survival kinase Akt.

This dysbalance of p-Syk/p-Akt appeared to be likely related to an enhanced tyrosine phosphatase activity in these patients. The disturbed CD22 expression and imbalanced equilibrium of kinases and phosphatases possibly led to a dysbalance of p-Syk and p-Akt resulting in an enhanced survival of SLE B cells. These observations were independent of *in vivo* pre-activation or environmental conditions.

Furthermore, a reduced basal expression of Syk and p-Syk expression was found in SLE B cells that could influence the BCR response in SLE patients. However, SLE patients expressed an enhanced frequency of a unique memory like CD27-Syk++ population, which showed a heterogeneous recently activated memory-like phenotype. Besides the lack of CD38 and an enhanced level of p-Syk, CD19, CD20 expression, memory characteristics like mutated $V(D)J_H$ gene rearrangements and largely absent ABC-B1 transporter activity was found. While *in vivo* immune activation by a secondary tetanus toxoid vaccination as well as follow-up studies in SLE patients did not find a relation with immune or disease activation, respectively. *In vitro* stimulation studies showed an INF-γ and TNF-α dependent generation of CD27-Syk++ B cells in patients with SLE. Further, a memory-like p-Syk and p-Akt phosphorylation kinetic after BCR

activation and the ability to differentiate in (auto)-Ab producing plasma cells were observed.

Noteworthy, CD27+, CD27-Syk+ and the novel memory-like CD27-Syk++ B cell population showed no differences in the ratio of p-Syk/p-Akt upon BCR engagement indicating a general B cell activation abnormality in patients with SLE. Further studies are needed to delineate if the p-Syk/p-Akt dysbalance is rather intrinsic or in response to an overly activated immune system in SLE.

5. Discussion

The current study analyzed in detail BCR related intracellular signaling pathways in patients with SLE compared to HD. In more detail, potential disturbances in the BCR signaling leading to the development and maintenance of autoreactive B and plasma cells were investigated in this prototypic systemic autoimmune disease.

The current findings provide evidence that SLE patients display an increased tyrosine phosphatase activity and an imbalanced CD19/CD22 expression ratio that lead to a diminished Syk and PLC-γ2 activation and further downstream to a decreased Ca^{2+} influx. Otherwise, a hyperactive Akt phosphorylation after BCR stimulation in these patients was found and excluded the possibility that there is a globally reduced intracellular kinase activity in lupus B cells. The disturbed balance between p-Syk and p-Akt towards the pro-survival kinase Akt may be involved in the circumvention of BCR induced negative selection together with enhanced survival of B cells and increased differentiation into plasma cells in patients with SLE.

Additionally, whole blood analysis of SLE patients revealed a diminished Syk and p-Syk(Y352) expression in CD27+ and CD27- B cells and unfolded a hitherto unknown Syk++ B cell subset lacking the conventional memory marker CD27. This study could demonstrate that the use of this intracellular marker is able to detect also other previously described CD27- memory B cell subsets but appear to be superior compared to previous cell surface phenotyping. CD27-Syk++ B cells showed an activated memory-like phenotype with the ability to differentiate into auto-Ab secreting B cells and displayed an increased tonic BCR signaling represented by enhanced basal expression of Syk and p-Syk. This data provide evidence, that certain intracellular mechanisms are not sufficiently in place or able to control these cells which could also play a key role in the maintenance of autoreactivity.

5.1 Intrinsic molecular defects in SLE patients lead to the development and maintenance of autoimmunity

5.1.1 Enhanced expression and activation of CD22 could lead to the reduced Syk and PLC-γ2 phosphorylation observed in SLE B cells

This study was set out to address whether BCR signaling events of SLE B cells are abnormal and may explain the importance of B cell effector mechanisms in SLE, as a disease with abundant autoimmune findings, including ANA induction.

First, analyzing the expression and activation of the inhibitory co-receptor CD22 as well as the balance between CD19/CD22 revealed enhanced CD22 expression on CD27- compared to CD27+ B cells in SLE and HD. Interestingly, CD27- B cells of SLE patients showed increased CD22 expression and phosphorylation kinetics after BCR stimulation as well as a disturbed CD19/CD22 balance compared to HD. However, no differences between SLE and HD B cells have been observed comparing the recruitment of SHP-1 to CD22 or CD22 to the activated BCR complex indicating that there are no defects in the signalosome formation but rather signaling disturbances.

Consistent with the publication of Daridon et al. (106) we could show, that naïve CD27- B cells have elevated expression levels of CD22 compared to CD27+ memory HD B cells. Contrary to our findings, Liossin, Suzuki and colleges found similar expression levels of CD22 and CD19 in SLE compared to HD B cells (85, 163). However, they did not differentiate between CD27+ and CD27- B cells and may have overlooked the reduction on CD27- SLE B cells especially since the frequency of CD27+ B cells is significantly enhanced in these patients (134).

An increased expression and activation of CD22 on B cells would induce an enhanced activation of the CD22 associated phosphatase SHP-1 which in turn would lead to a reduced activation of Syk and PLC-γ2 after BCR activation as it has been described by Sieger et al. (111). Confirming these findings, SLE B cells displayed a diminished B cell response after BCR stimulation as shown by a decreased Syk and PLC-γ2 phosphorylation, including reduced Syk phosphorylation efficiency as well as a diminished Ca^{2+} mobilization.

In accordance with our findings, a decreased expression level of the positively regulating BCR co-receptor phosphatase CD45 has been reported (5, 196) which could lead to a diminished B cell response in patients with SLE. Additionally, a decreased p-Syk and p-PLC-γ2 response in IgM+ and transitional B cells (197) as well as reduced

levels of p-BLNK were observed after IgM stimulation in patients with SLE (198). Furthermore, reduced BLK expression in mouse and humans due to genetic defects were detected leading to a diminished BCR response and enhanced frequency of autoreactive naïve B cells, increased expression of pro-inflammatory cytokines and enhanced susceptibility for autoimmune kidney disease (174, 199). In mice models carrying the lupus-associated Ly108.1 allele, a rather diminished BCR response with a reduced proliferation capacity and apoptosis have been predicted to be involved in SLE. Ly108 (CD352) is a member of the signaling lymphocytic activation molecule (SLAM) family receptors and is important during B cell tolerance (175). This polymorphism led to a disturbed GC response, auto-Ab production, reduced Ca^{2+} influx after IgM stimulation and breakdown of tolerance (175, 200). Especially the reduced Syk activation and further the decreased Ca^{2+} influx could favor the development of self-reactive B cells since Cappione et al. could show a reduced Ca^{2+} response in autoreactive B cells (156).

Interestingly, challenging SLE patients with standard immunizations or stimulating lupus B cells with polyclonal activators *in vitro* resulted in substantially decreased amounts of specific Ab and a reduced immune response (201). In this context, Sieber et al. could recently show a rather hyporesponsive SLE B cell phenotype indicated by a decreased cytokine production of IL-6 and IL-10 after TLR9 stimulation (202). These results appear to be contradictory to the widely accepted B cell hyperactivity based on the induction of various auto-Abs (203), detection of increased plasmablasts in the circulation of SLE patients (134, 140, 204) and spontaneous development of Ig secreting cells *in vitro* (141, 142, 205, 206).

Contradictory and implicating a rather hyperactive B cell phenotype, a number of studies described an enhanced tyrosine phosphorylation and Ca^{2+} influx as well as an increased p-Syk response in $CD19^{high}$ (136, 137), CD27+ and CD27- SLE B cells due to a reduced Lyn expression (163) and lipid raft localization (164), reduced expression of the FcγRIIb receptor (168) or enhanced occurrence of INF-α (6). The results suggest so far a globally enhanced tyrosine phosphorylation in SLE B cells that includes all kinases and does not dissect single ones like Syk or PLC-γ2. However, this hypothesis needs to be proven. Therefore, a detailed analysis of BCR downstream kinases is needed to differentiate between a hyper- and/or hyporesponsive B cell phenotype in SLE. The differences concerning the Ca^{2+} influx could be due to the fact that PBMCs were stimulated with IgM only or enriched B cells were stained against CD20 prior to IgM stimulation whereas untouched negative selected B cells were used in this experimental

setup. Since CD20 is known to be involved in Ca^{2+} signaling as a potential Ca^{2+} channel (207, 208) using an anti-CD20 antibody to label B cells could pre-activate these cells and influence the Ca^{2+} response. The variations regarding p-Syk in CD27+ (137) and CD27- CD38++ transitional B cells (6) could be explained by the fact that the percentage of responding cells (% of p-Syk) after IgG or IgM stimulation was analyzed in SLE in contrast to the MFI values used in this study.

Collectively, our results provide evidence that the enhanced expression and activation of CD22 as well as the disturbed balance of CD19/CD22 on CD27- SLE B cells could potentially lead to an enhanced activation of the CD22 associated tyrosine phosphatase SHP-1 and thereafter to an enhanced dephosphorylation of Syk and diminished BCR response in SLE B cells. Interestingly, this diminished BCR response was observed in CD27- but also CD27+ SLE B cells even though CD27+ B cells did not show any abnormalities with regard to CD22. One explanation could be that CD27+ SLE B cells display an enhanced tyrosine phosphatase activity either directly related to SHP-1 or to other phosphatases leading to an enhanced dephosphorylation of Syk independent of the expression level or activation of CD22.

Overall the data shed new light in the role of B cell autoimmunity and put forward the hypothesis that an intracellular signaling dysbalance may account for autoimmune B cell emergence and maintenance in SLE.

5.1.2 Similar BCR response of CD27- and CD27+ B cells and isotype independent diminished Syk activation in SLE patients

Comparison of the BCR response between CD27+ and CD27- B cells showed in HD but not in SLE patients an enhanced activation capacity of CD27+ compared to CD27- B cells. In line with our findings, other groups described a more responsive phenotype in HD CD27+ memory compared to CD27- B cells indicated by a stronger formation of BCR oligomers, microcluster formation, higher phosphorylation kinetics and a more effective recruitment of kinases (75). Intrinsic differences, such as the expression of genes involved in activation, co-stimulation and survival as well as the decreased expression of the inhibitory BCR co-receptor CD22 are responsible for the more efficient BCR response in CD27+ B cells (77, 106). However, the phosphorylation of Syk and PLC-γ2 in CD27+ versus CD27- B cells in SLE patients has not been analyzed in greater detail so far. Thus, the same BCR response of CD27+ and CD27- SLE B cells leave room for various

speculations. On the one hand, the chronic immune activation observed in patients with SLE (1) could lead to a less responsive phenotype of CD27+ SLE B cells. On the other hand, aberration in co-receptor expression like CD19, CD21 or CD81 which positively regulate BCR signaling (113, 138, 176, 209) could result in a disturbed BCR response. Therefore, further analyses are needed to reveal which signaling abnormalities especially related to CD27+ memory SLE B cell are effective in SLE.

Additionally, analysis of the frequency of CD27+IgM+ and CD27+IgG+ memory B cells in SLE and HD showed no differences, precluding, that the differences regarding the BCR response between SLE and HD B cells is not due to an enhanced occurrence of less responsive CD27+IgM+ memory B cells (76) or related to Ig-switched B cells in patients with SLE. This suggests that these abnormalities are rather of intrinsic nature and also isotype independent.

We can conclude, that the diminished Syk response observed in SLE B cells is found in CD27-, CD27+IgM+ as well as CD27+IgG+ B cells with a more pronounced amplitude in CD27+ memory B cells and suggest an intrinsic SLE associated disturbance.

5.1.3 *In vivo* pre-activation does not correlate with the diminished Syk response in SLE B cells

A chronic *in vivo* immune activation can lead to an exhausted and non-responsive phenotype as it has been described for T cells (210, 211). Especially CD27- naïve B cells in SLE patients have been shown to be antigen engaged and as a consequence pre-activated *in vivo* (180). Therefore, the occurrence of CD80+ and CD86+ B cells in SLE patients were analyzed. Indeed, an increased frequency of CD80+ and CD86+ B cells in SLE patients was observed indicating an *in vivo* pre-activation of circulating B cells in these patients as it has been observed already by other groups (180, 181, 212). However, correlation analysis between the frequency of these cells and Syk phosphorylation after BCR activation showed no interrelation. This observation makes it unlikely, that the *in vivo* pre-activation is responsible for the diminished Syk response in SLE B cells. Confirming this data, an *in vitro* resting phase for 24 or 48 h and the removal of the pro-inflammatory environment including SLE-related enhanced INF-α levels and other inflammatory cytokines/mediators (120, 149, 182-184, 213), did not restore the BCR response or the missing differences between CD27- and CD27+ B cells in SLE patients.

This data, together with the absence of a correlation between remission, disease activity or therapeutic interventions suggest that intrinsic isotype independent defects induce the disturbed BCR response observed in SLE B cells.

5.1.4 Hyperactivation of the serine kinase Akt after BCR stimulation in SLE

Analyzing the activation of the pro-survival kinase Akt after BCR stimulation showed increased phosphorylation kinetics in CD27+ and CD27- SLE B cells compared to HD. Thus, it is unlikely that there is a global diminished kinase phosphorylation capacity or a non-responsive phenotype in SLE B cells due to chronic antigen stimulation or therapeutic interventions. In line with our findings, multi-signaling analysis in lupus mice revealed enhanced activation of the PI3K/Akt signaling pathway (162). Additionally, Taher et al. showed enhanced levels of PI3K and phosphorylated Akt in B cells of SLE patients (214). Moreover, Wu and colleges described an enhanced expression of PTEN in CD27- SLE B cells leading to hyperactivation of Akt (165). Of particular note, enhanced PI3K and therefore Akt activation in B cells led to an increased plasma cell formation and reduced class-switch recombination reflecting the hyperactive B cell phenotype as described in the literature, including auto-Ab production, enhanced frequencies of circulating plasmablasts and spontaneous secretion of Ab *in vitro* (105, 141, 142, 205, 206, 215).

We conclude that the increased Akt phosphorylation in CD27- and CD27+ SLE B cells after BCR activation could lead to the elevated generation of plasmablasts/plasma cells observed in SLE (36, 135, 140). Furthermore, the enhanced differentiation into plasma cells due to elevated Akt activation could be responsible for the reduced frequency of periphery B cells in SLE patients (lymphopenia) (216).

5.1.5 Disturbed balance of p-Syk/p-Akt in B cells of patients with SLE could lead to the breakdown of self-tolerance

As discussed earlier, hypo- as well as hyperactive B cell responses were found in SLE dependent on the kinase analyzed. Similar results were obtained in this study. While the BCR response regarding Syk and PLC-γ2 was diminished, the activation of Akt was increased in SLE B cells. Therefore, a general prediction whether B cells of SLE patients suffer from an enhanced or a diminished B cell signaling response cannot be concluded

at this time. It appears that the interactions and the balance of BCR related signaling kinases are important and determine the B cell fate and development of autoreactivity.

In line with this, comparing the phosphorylation of Syk and Akt in CD27+ and CD27- SLE and HD B cells revealed an imbalanced signaling network which could lead to the survival of autoreactive B and plasma cells as described in the literature (36, 135, 140, 165). In fact, the current study could show that SLE patients had a reduced p-Syk/p-Akt ratio compared to HD B cells towards the pro-survival kinase Akt which was correlating with the expression of CD19/CD22 in CD27+ and CD27- SLE B cells. In line with this, the balance between CD19/CD22 has been already associated to the development of autoimmune diseases (84, 115, 116). However, the direct impact on the signaling outcome after BCR activation in SLE versus HD B cells has not been investigated so far. Our data indicate that the balance between CD19 and CD22 has a direct effect on the activation strength and therefore ratio of p-Syk and p-Akt. This could be an explanation for the reduced Syk phosphorylation in CD27+ B cells even without showing enhanced expression or activation of CD22.

Furthermore, Syk-/- mice fail to enter the re-circulating follicular B cell pool indicating that Syk plays a crucial role during positive selection processes within the GC reaction (59). Together with the findings, that the strength of the BCR response determines the B cell fate (16, 56, 217), the disturbed balance of p-Syk/p-Akt in CD27- as well as CD27+ SLE B cells could be responsible for the lack of sufficient central and peripheral selection of autoreactive B cell clones and could contribute to the emergence of autoimmunity and breakdown of self-tolerance.

5.1.6 Enhanced tyrosine phosphatase activity leads to the dysbalanced activation of Syk and Akt in SLE B cells

The activation of kinases is counterbalanced by phosphatases and is important for the BCR signaling strength as it has been shown for the Syk/SHP-1 (90) and Btk/SHIP-1 equilibrium (218). In the study of Alsadeq et al. Syk and SHP-1 single deficient compared to double knockout mice were analyzed. Syk single knockout mice showed a developmental block during the early B cell phase, whereas SHP-1-/- mice had enhanced frequencies of B1 transitional B cells and developed an autoimmune phenotype. The double knockout phenotype (Syk-/- SHP-1-/-) could partially rescue the maturation of B cells but displayed defects in BCR but not TLR or CD40L induced signaling. Thus, the

equilibrium between Syk and SHP-1 might be crucial for normal B cell development and BCR signaling (90). A similar conclusion was made by Liu and colleges; Btk deficient mice showed a reduced BCR aggregation and actin remodeling compared to wild-type B cells leading to a reduced B cell activation. In SHP-1-/- B cells a normal BCR aggregation compared to wild-type B cells was observed. However, the BCR aggregation persists over a longer time period inducing higher levels of BCR signals. Regarding the opposing effect of Btk and SHIP-1 deficiency, an important balance between these two molecules needs to be taken into consideration (218).

Therefore, the tyrosine as well as serine/threonine phosphatase activities were analyzed in SLE B cells. Interestingly, SLE B cells showed an enhanced tyrosine but similar serine/threonine phosphatase activity compared to HD. Furthermore, inhibition of the tyrosine phosphatase activity was efficient to overcome this effect and increases Syk phosphorylation with an impact on the p-Syk/p-Akt ratio after BCR activation in CD27+ and CD27- SLE B cells.

In line with our data, enhanced levels of the tyrosine phosphate PTPN22 was observed in SLE T and B cells (169, 171, 219). In contrast, an increased expression of the serine/threonine proteinphosphatase 2A (PP2A) in SLE mouse T cells was reported leading to increased IL-17 production and higher occurrence of lupus nephritis (220). Since we analyzed the global phosphatase activity, it cannot be excluded that specific serine/threonine phosphatases are also reduced in SLE B cells. Additionally, a reduced expression level of the tyrosine phosphatase SHP-1 in SLE B cells has been reported (5). Due to the fact, that only the expression and not the phosphatase activity of SHP-1 was analyzed, the reduced SHP-1 level in SLE B cells does not preclude that SLE patients show a higher phosphatase activity which would lead to an enhanced dephosphorylation of Syk.

Of note, it has been published that GC B cells have a diminished signaling strength compared to naïve and non-GC B cells which was due to an enhanced phosphatase activity. Interestingly, GC B cells showed hyperphosphorylation of the tyrosine phosphatase SHP-1 (177). For CLL B cells, a reduced activation of Syk, BLNK and PLC-γ2 after IgM stimulation dependent on increased phosphatase activity was observed (221).

Our data lead to the conclusion that the phosphatase activity directly regulates the BCR response and reinforces the hypothesis that the enhanced tyrosine phosphatase activity observed in SLE B cells led to the shifted balance between the tyrosine kinase

Syk and the serine kinase Akt. However, it has to be further investigated which specific phosphatase or a certain group of phosphatases are involved in this process.

5.1.7 Dysbalanced activation of Syk and Akt led to enhanced survival of SLE B cells

Analyzing the viability of HD and SLE B cells after BCR activation revealed an enhanced survival of SLE B cells. Furthermore, the frequency of viable cells strongly correlated with the ratio of p-Syk/p-Akt in SLE B cells indicating that an enhanced activation of Akt led to increased survival of these cells.

In line with our data, overexpression of the tyrosine phosphatase PTPN22 in CLL B cells led to the blockade of IgM induced apoptosis but positively regulated the anti-apoptotic kinase Akt showing the same imbalance of signaling pathways observed for SLE in this study (95, 170). Accordingly, it was published that enhanced activation of PI3K contributes to enhanced survival of memory T cells in SLE. However, contradictory results were published regarding the survival of lymphocytes in SLE patients. Some found less survival of lymphocytes or increased expression of the death receptor CD95 (Fas) on B cells in SLE patients (222, 223). Others found increased T cell but not B cell apoptosis in SLE (224) and an enhanced survival of transitional B1 cells in mice after IgM stimulation due to an enhanced expression of the anti-apoptotic transcription factor Bcl-2 (225). Furthermore, the occurrence of elevated levels of BAFF (57, 89, 128), known to be anti-apoptotic, indicates a rather enhanced survival of SLE B cells. In line with this, the reduced expression of the PLC-γ2 downstream kinase PKCδ in patients with SLE led to a resistance against apoptosis after BCR activation (226, 227). Interestingly and in accordance with a reduced Lyn expression in SLE patients, a targeted deletion of Lyn induces increased Akt activation in antigen stimulated B cells and further downstream to an enhanced B cell survival (228). The differences between the reduced survival found in the literature and the enhanced survival observed in this study could be explained by the fact that mainly lymphocytes (222, 223) and the spontaneous BCR independent apoptosis was analyzed (224).

Taking together, the dysbalanced activation of Syk and Akt in SLE B cells appears to be related to an enhanced tyrosine phosphatase activity and a disturbed CD19/CD22 expression that could lead to the circumvention of selection checkpoints at certain stages, including the BM as well as in the periphery. As consequence, a preferential differentiation

of autoreactive B cells which would have been normally negative selected and may result into an increased survival of (autoreactive) plasma cells.

5.1.8 Reduced expression of Syk and p-Syk in SLE B cells

Analysis of the baseline expression of Syk and p-Syk(Y352) in untouched whole blood B cells from SLE patients and HD revealed a significant reduced expression of Syk in CD27+ and p-Syk(Y352) in CD27+ and CD27- SLE B cells.

In contrast with this data, Iwata and colleges found increased Syk and p-Syk(Y348) levels in unstimulated CD19+ B cells of SLE patients (102). This variation could be explained by the fact, that CD19+ SLE B cells were not separated by their CD27 expression. Furthermore, they analyzed the frequency of p-Syk positive B cells purified by Ficoll on tyrosine 348 (102). However, we determined the MFI values of p-Syk on the tyrosine phosphorylation site 352 using untouched whole blood CD27+ and CD27- B cells.

Together, the enhanced tyrosine phosphatase activity, the dysbalanced CD19/CD22 expression and the reduced expression of Syk and p-Syk observed in this study could be responsible for the reduced Syk phosphorylation efficiency and overall reduced Syk and PLC-γ2 activation in SLE B cells.

5.2 Enhanced frequency of a novel CD27-Syk++ B cell subset in patients with SLE could contribute to the maintenance of autoreactivity

Besides detailed signaling abnormalities in SLE B cells, a new CD27- population expressing high levels of Syk and p-Syk was identified in these patients. Interestingly, no correlation between the SLEDAI, the ANA-titer or therapeutic interventions was found. Furthermore, the occurrence of CD27-Syk++ was stable over time. In contrast, abnormalities in SLE associated B cell subsets were found to correlate with the SLEDAI as it has been shown for CD27++ plasma cells (140) or CD27-IgD-CD95+ memory B cells (36). Our data indicate, that the generation of CD27-Syk++ is due to intrinsic defects as it has been shown for CD27+ and CD27- SLE B cells in this study or environmental abnormalities related to SLE which are not necessarily dependent on the activity status. Disturbed cytokine production and signaling aberrations have also been found in SLE patients (5, 98, 99, 120, 122, 126, 129, 131, 153, 198, 229, 230) and could be – at least partially - responsible for the generation of SLE associated B cell subset abnormalities.

5.2.1 B cell subset independent BCR associated intrinsic defects

To analyze if the enhanced expression of Syk would lead to a hyperactive BCR response, the Syk phosphorylation kinetic in CD27-Syk+, CD27-Syk++ and CD27+ SLE B cells in contrast to controls was analyzed. Interestingly, even though enhanced basal Syk and p-Syk levels were observed in CD27-Syk++ B cells, the BCR response regarding p-Syk was comparable to CD27+ SLE memory B cells but still diminished compared to CD27+ HD memory B cells.

This observation indicates, that CD27-Syk++ might display the same enhanced tyrosine phosphatase activity which downregulates the BCR response as it has been observed for SLE B cells. Overall, the ratio of p-Syk and p-Akt was the same in CD27-Syk++, CD27-Syk+ and CD27+ B cells indicating a general and rather intrinsically and disease activity independent abnormality of Syk regulation in SLE B cells.

5.2.2 CD27-Syk++ represent a unique memory-like B cell subset

Comparison with previously published B cell abnormalities in SLE including CD27+/-CD21- (138), transitional CD19+CD24++CD38++ (231), CD27+/-CD19^{high} (136), CD27-IgD- (33) and CD27-IgD-CD95+ (36) B cells showed similarities but no complete overlap. The lack of CD38 of CD27-Syk++ B cells excludes the conformity with CD27-CD24++CD38++ transitional B cells. Moreover, CD21- and CD19^{high} B cells comprise CD27- as well as CD27+ B cells and CD27-IgD- are precluding CD27-Syk++IgD+ B cells. Additionally, not all analyzed B cells subset aberrant in SLE were Syk++. Therefore, CD27-Syk++ reflect a B cell subset predominantly enhanced in SLE which has not been described so far.

Phenotypic analysis of CD27-Syk++ revealed characteristics which were distinct from classical naïve and memory B cells, including a higher expression of CD19 and CD20, increased frequency of CD21- and diverse frequencies of IgM+ only and IgM-IgD- class-switched B cells. However, conventional memory characteristics were consistently observed for these cells, such as the lack of an active ABC-B1 transporter and the lack of expression of CD38, imprints of SHM within V(D)J regions and class-switch as well as similar frequencies of CD95+ B cells. Based on these findings, the overall data are consistent with the conclusion that CD27-Syk++ B cells represent a unique

heterogeneous pre-activated B cell subset with memory characteristics, including IgM+ but also class-switched IgG+ and IgA+ memory B cells.

These results are consistent with the conclusion that intracellular Syk can serve for B cell abnormalities in SLE patients as well as a reliable marker for the identification of activated B cells with memory characteristics, including certain CD27- subsets.

5.2.3 Accumulation of Syk in CD27-Syk++ B cells could be due to an aberrant degradation of Syk

In the current study, confocal analysis showed an elevated cytoplasmic accumulation of Syk in CD27-Syk++ compared to CD27+ and CD27-Syk+ B cells. Defects in apoptosis and proteasomal degradation have been already described in SLE, which would influence the cellular distribution and accumulation of Syk within the cytoplasm. However, since neither CD27+ nor CD27-Syk+ B cells were similarly affected by proteasomal abnormalities, such an explanation appears to be less likely. The increased tyrosine phosphatase activity observed in SLE B cells could lead to an increased generation of Syk in CD27-Syk++ B cells and therefore reflects a mechanism to compensate the reduced Syk phosphorylation response observed in these patients.

Furthermore, aberrations of the ubiquitin-protease system tightly controlled by the ubiquitin ligase Cbl could lead to the dysfunction of the homeostasis and defects in Syk degradation (91-93). Interestingly, studies in human myeloblastic leukemia and monocytic cells have shown that the activation of CD38 is important to activate Cbl which in turn would degrade Syk (232, 233). Since CD27-Syk++ B cells have been described as CD38-, lacking this receptor could lead to an inefficient activation of Cbl and degradation of Syk. To confirm such interrelation, further studies are necessary to clearly link the absence of CD38 with the accumulation of Syk in the cytoplasm.

5.2.4 Heterogeneity of CD27-Syk++ B cells is due to the origin by different developmental pathways

Since CD38 is downregulated on class-switched CD27+ memory B cells after GC reaction (234) one possibility could be, that CD27-Syk++ B cells undergo multiple rounds of GC reactions and downregulate or shed CD38 as well as CD27. Additionally, CD38 is necessary for the recruitment and development during GC reactions (235) indicating that

CD27-Syk++ B cells lacking CD38 would undergo less stringent extrafollicular immune reactions, give rise to IgM+ memory B cells and fail to upregulate CD27.

5.2.4.1 GC dependent development

Speaking in favor for a GC dependent generation of CD27-Syk++ B cells, IgG+ and especially IgA+ showed a high frequency of SHM within V(D)J regions indicating that they have undergone a striking GC reaction as it has been already described for SLE B cells (157, 236). In fact, 5 out of 7 analyzed CD27-Syk++IgA+ clones showed a mutation frequency of over 9% (range 9-20%). Therefore, the lack of CD27 could be explained by a strong GC reaction which would lead to the loss or downregulation of CD27. In fact, an intense GC activity led to the generation of recently activated CD27-IgD-CD95+ memory B cells (36). It is noteworthy that similar observations were reported for T and B cells in HIV patients, elderly people and after a chronic humoral immune activation where class-switched memory B cells do not express the memory marker CD27 (32, 37, 210, 211, 237). In line with this, Jung et al. found that B cells acquire CD27 at the centroblast stage and lose it gradually upon further differentiation (31). Since no correlation between the concentration of sCD27 and the frequency of CD27-Syk++ B cells has been found in our study, an increased shedding of surface CD27 is rather unlikely and would suggest that CD27-Syk++ cells downregulated CD27 or do not upregulate this marker at the first place. However, since the frequency of B cells within the whole blood represents only a minor fraction, the detection sensitivity could be insufficient to identify minor changes of B cell specific sCD27.

5.2.4.2 Extrafollicular development

The highly mutated CD27-Syk++IgG+ and IgA+ clones, 3 of 5 CD27-Syk++IgM+ clones showed no SHM within V(D)J regions and 2 clones a mutation frequency of 2 and 8% indicating that CD27-Syk++IgM+ developed during GC or even T cell independent and rather extrafollicular immune reactions. In line with this, approximately 70% of CD27-Syk++ B cells express IgM and only 30% were class-switched. It is well established, that an extrafollicular response would lead to the generation of low mutated IgM+ memory B cells which have not yet upregulate CD27 (51, 52, 238). Indeed, extrafollicular structures were often found in autoimmunity and may contribute to local production of

auto-Ab (238) as it has been observed for CD27-Syk++ B cells by the differentiation into (auto)-ASCs.

Therefore, CD27-Syk++ B cells might develop during GC dependent and independent as well as T cell independent and dependent immune reactions and could develop into short-lived and less mutated IgM as well as long-lived highly mutated class-switched memory B and plasma cells.

5.2.4.3 Progeny or progenitor of CD27+ memory B cells

An alternative possibility is that CD27-Syk++ cells represent not only progenitor of CD27+ memory B cell which have not yet acquired CD27. These cells could also represent progeny of CD27+ memory B cells due to the loss of CD27 after GC reaction and immune activation. Confirming the assumption that CD27-Syk++ B cells would represent progeny of CD27+ memory B cells, 70% of these cells to not express CD21. The lack of the complement receptor CD21 has been associated to chronic immune activation leading to a status called “exhaustion” (210). Together with the reduced generation of CD27++ B cells after 5 days *in vitro* culture compared to conventional CD27+ memory B cells it can be assumed that a persistent immune activation could lead to the emergence of CD27-Syk++ B cells lacking CD21. Interestingly, INF-α has been found to be a ligand for CD21 (209). Therefore, enhanced levels of INF-α in patients with SLE (149, 213) would continuously stimulate SLE B cells via CD21 and other conventional receptor pathways leading to the downregulation of this receptor on CD27-Syk++ B cells. In line with this, analyzing the phosphorylation of Akt and Syk after BCR engagement showed memory-like phosphorylation kinetics similar to CD27+ B cells even though CD27-Syk++ B cells expressed significantly more Syk. This data indicate, that CD27-Syk++ B cells show a reduced Syk phosphorylation efficiency which would speak in favor for an “exhausted” phenotype. However, the balance between p-Syk and p-Akt was similar to the balance observed for CD27+ and CD27-Syk++ B cells and is contradictory to this assumption. Additionally, no correlation between age and the frequency of CD27-Syk++ B cell in HD was observed which would argues against an “exhausted” or better post-activated phenotype.

5.2.5 Increased basal expression of Syk and p-Syk, the lack of CD27 and imbalanced p-Syk/p-Akt ratio could lead to an enhanced survival of CD27-Syk++ B cells

CD27-Syk++ B cells display an enhanced accumulation in the cytoplasm and basal phosphorylation of Syk reflecting an increased tonic signaling in these cells. The tonic signaling of B cells is essential during B cell development, selection processes and survival (17, 78). Therefore, CD27-Syk++ B cells could display increased survival capacity and therefore playing a key role in the maintenance of autoreactivity in SLE. Accordingly, co-ligation of CD27 at the same time with the BCR induces CD27- mediated apoptosis (28) and could implicate together with the enhanced activation of the pro-survival kinase Akt, that Syk++ cells lacking CD27 represent a B cell subset increased in SLE which is more resistant against cell death. In line with this, defects in apoptosis are widely known in SLE (222-224) and appear to be consistent with this hypothesis.

5.2.6 CD27-Syk++ could represent a tissue resident B cell subset

Of importance, a current report described that CD27-IgG+FcRH4+ memory B cells reside within tonsil and possibly other secondary lymphoid organs (39) suggesting that the appearance of CD27-Syk++ B cells in the circulation of SLE patients may reflect a GC spillover of this population into the periphery due to an increased GC activity and memory B cell induction or processing. Alternatively, homing or entrance of this population within secondary lymphoid organs may be altered in SLE leading to their persistence in the blood. Therefore, further analysis regarding the expression of homing receptors like integrin ß7 (239), CXCR5 (240, 241) or CXCR4 (241) on CD27-Syk++ B cells are needed to further delineate the origin of these cells. Further characterization of the underlying mechanisms involved may direct or redirect therapeutic interventions.

5.2.7 Microenvironmental conditions induce the generation of CD27-Syk++ B cells

However, not only intrinsic B cell abnormalities may require considerations, since dysfunction of other immune cells, like T cells, DCs and macrophages have been associated with SLE (4, 99, 153, 242-244) and might have an impact on secondary B cell abnormalities. Especially disturbances in cytokine expression by these cells were noted (120, 126, 131, 229, 245) and could influence the development and activation of aberrant

B cell populations in SLE patients. Accordingly, *in vitro* analysis showed that the dysregulated cytokine environment in SLE patients could play an essential role during the development and maintenance of CD27-Syk++ B cells. *In vitro* stimulation with the anti-inflammatory cytokines INF-γ, TNF-α as well as the stimulation with LPS but not INF-α, IL-6, IL-21 or BAFF led to an increased frequency of Syk++ B cells. Confirming these observations, studies have shown, that TNF-α regulates the expression and activation of Syk (246). Interestingly, immunization with the tetanus toxoid did not induce the generation of CD27-Syk++ cells indicating a cytokine involvement which is rather Th1 (e.g. INF-γ and IL4) dependent (41). It is not clear if the vaccination with tetanus toxoid induces exclusively or predominantly a Th2 response (247-249). Therefore, an exact conclusion if the development of CD27-Syk++ B cells is Th1 or Th2 (e.g. INF-α, IL-6) driven cannot be drawn at this time. Interestingly, Akahoshi et al revealed a disturbed balance of Th1/Th2 towards Th1 cytokines in SLE patients (250). Additionally, increased levels of serum INF-γ in human SLE patients (251) as well as in MRL-*Fas*^{lpr} mice have been reported (252). Of note, analysis of HD with different inflammatory diseases revealed that healthy controls with dental root inflammation can also carry an enhanced frequency of CD27-Syk++ B cells. If this dental roots infection induces a Th1 response rather than a Th2 response has to be further investigated. However, this findings should not be taken as evidence that Th2 cytokines do not influence or regulate the occurrence of CD27-Syk++ B cells in SLE patients.

No correlation between CD80+ and CD86+ B cells with the frequency of CD27-Syk++ B cells in SLE was found. Therefore, a general immune activation seems to be unlikely related with the generation of CD27-Syk++ B cells. Therefore, a combination of intrinsic abnormalities as well as environmental factors including a disturbed cytokine milieu are probably involved or responsible for the emergence of CD27-Syk++ B cells.

Together, CD27-Syk++ B cells reflect an activated B cell subset with memory characteristics which can be generated by different maturation pathways and could reflect progenitor as well as progeny of CD27+ memory cells in patients with SLE. Furthermore, the disturbed cytokine environment and the lack of CD38 expression in SLE patients could favor the accumulation of Syk in these cells, which would potentially lead to an enhanced tonic BCR signaling and bypass of conventional differentiation pathways as well as survival and emergence of autoreactive B and plasma cells. Alternatively, the

increased Syk expression could reflect a compensation mechanism for accelerated phosphatase activity observed in this study for SLE B cells.

However, further studies are needed to reveal the nature of the emergence/induction and maintenance of these cells and how they are involved in lupus autoimmunity.

5.3 Conclusion and outlook

The initial question of this thesis was to analyze how B cell tolerance in SLE patients is disturbed and how autoreactive B cell clones survive and maintain in the repertoire of this disease. To which extent abnormalities of B cell subsets, intracellular BCR signaling pathways and BCR complex organization are involved in this process could be partially answered in this study.

The current thesis provide evidence that SLE B cells display a dysbalanced BCR signaling (p-Syk/p-Akt) and abnormalities in the BCR complex organization (CD19/CD22) together with the occurrence of CD27-Syk++ B cells with BCR signaling comparable to conventional CD27+ lupus memory B cells. These abnormalities appear to be consistent with an enhanced capacity to differentiate into (auto)-ASC in SLE patients and could represent a possible mechanism to circumvent negative selection checkpoints in the BM and the periphery since CD27- and CD27+ SLE B cells are equally affected. Simultaneously, the data suggest that these abnormalities can lead to an enhanced survival and therefore persistence of (auto)-reactive B and plasma cells.

This assumption can be solidified by the observation that SLE B cells have a reduced Syk but enhanced Akt response after BCR activation due to an enhanced tyrosine phosphatase activity, reduced basal Syk and p-Syk expression and dysbalance of the BCR co-receptor complex CD19/CD22. This altered balance could lead to the activation of a different transcription factor- and gene expression profile which would shift the B cell fate decision to an increased survival of autoreactive B cell clones and potential elevated plasma cell formation (

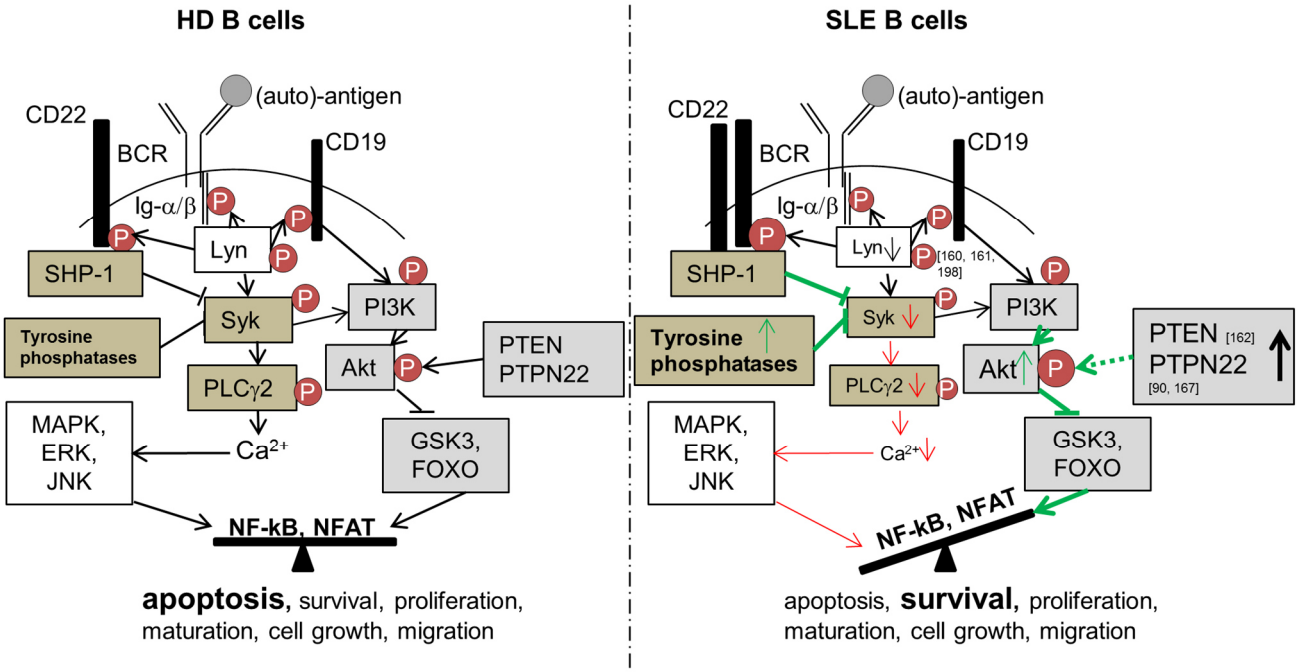

Figure 5-1).

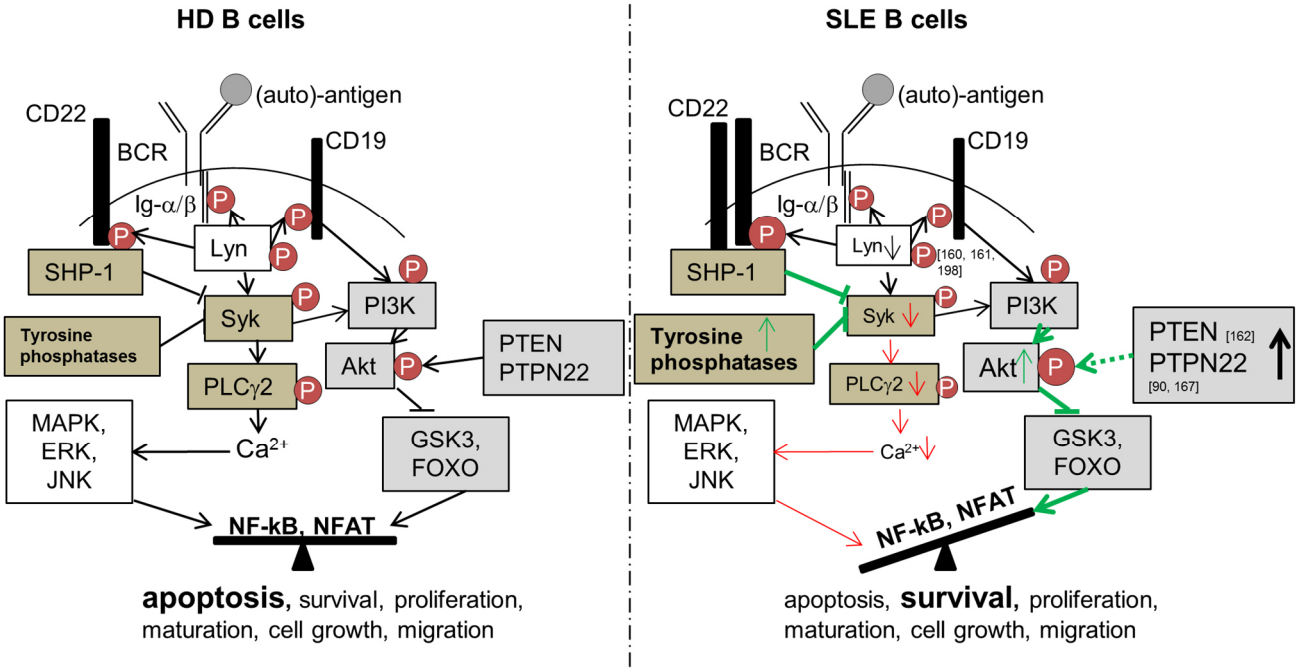

Figure 5-1: Proposed scheme of the dysregulated BCR signaling pathways in patients with SLE compared to HD. Schematic diagrams show the BCR signaling in HD **(A)** versus SLE patients **(B)**. (Auto)-antigen binding to the BCR induces the activation of Lyn which leads to an activation of several signaling pathways including Syk and CD19. On the one hand SLE B cells showed an enhanced CD22 phosphorylation that could lead to a reduced Syk activation as well as a reduced Ca^{2+} influx partially dependent on the balance between CD19 and CD22 expression as well as a global enhanced tyrosine phosphatase activity. On the other hand an enhanced activation of the PI3K/Akt signaling pathway in these patients was observed. This imbalance of the signaling strength of different BCR related signaling pathways could result in a disturbed relation between BCR dependent negative selection processes and impact of apoptosis that may permit a preferential survival of (auto)-reactive B and plasma cells under the conditions of SLE.

So far, a myriad on molecular aberrations in B cells of SLE patients including genetic defects, signaling abnormalities or aberrant expression profiles of receptors have been described (102, 152, 163, 167-169, 171, 175, 196, 199, 225, 226) but the balance between different signaling pathways have not been investigated in greater detail. In this regard, the observation of an imbalanced activation of Syk and Akt after BCR stimulation in SLE B cells reflects a hitherto unknown abnormality and could also be a novel therapeutic target. Therefore, specific inhibitors against PI3K or Akt have to be investigated regarding their ability to restore the balance between Syk and Akt. Also B cell specific Ab against BCR co-receptors, like the monoclonal Ab epratuzumab targeting CD22, need to be tested concerning its capacity to regulate this equilibrium. A modulatory effect of epratuzumab on Syk and PLC-γ2 is already known (111) but to which extent the PI3K/Akt pathway and therefore cell survival is influenced has not been investigated yet. The role of Lyn and its phosphorylation status after BCR activation in SLE versus HD B cells are needed to complete the study regarding BCR downstream signaling disturbances in this disease. Further, it has to be analyzed in more detail which phosphatases (e.g. SHP-1) or other signaling pathways (e.g. TLR9, CD40, BAFF, CD19) and BCR co-receptors (CD21, CD81) are involved.

Moreover, the unique CD27-Syk++ B cells enhanced in SLE represent a heterogeneous memory-like B cell subset containing activated IgM+, IgM+IgD+ and IgM-IgD- class-switched B cells. CD27-Syk++ B cells are probably generated through different maturation pathways and containing B cells of different developmental stages including progenitor as well as progeny of classical CD27+ memory B cells. CD27-Syk++ B cells could represent a tissue resident memory B cell subset which re-circulates in higher frequencies in the periphery of patients with SLE. Therefore it would be interesting to investigate the expression of surface markers like CD40, CD80 or MHC class II which are important during B-T cell interaction and antigen presentation as well as the expression of the homing receptors, CXCR4, CXCR5 or adhesion molecules, such as integrin beta 7. Other lymphoid organs like the spleen, tonsil or the BM needs to be analyzed regarding their occurrence of CD27-Syk++ B cells to understand more in detail the origin of these cells. However, since these B cells have been only found in humans so far, analysis regarding their tissue localization in human SLE patients is challenging.

In conclusion, the current study provides evidence that regulating the balance of BCR related signaling pathways might be a promising therapeutic target to modulate the development and persistent of autoreactive B cells in SLE. Additionally, the use of

intracellular markers, such as Syk, permits a more precise delineation of activated CD27- memory B cell subsets to understand the causes of memory in autoimmunity.

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Appendix

Table of figures

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Publication list

Fleischer SJ, Giesecke C, Mei HE, Lipsky PE, Daridon C, Dörner T.; "Increased frequency of a unique spleen tyrosine kinase bright memory B cell population in systemic lupus erythematosus." **Arthritis & Rheumatology 2014 Dec; PMID: 25156507**

Sarah J. Fleischer, Capucine Daridon, Vanessa Fleischer, Peter E. Lipsky, Thomas Dörner; "Disturbed balance of BCR related signaling pathways leads to an enhanced survival of B cells in patients with SLE." **Arthritis & Rheumatology March 2015; revision submitted**

Sieger N, Fleischer SJ, Mei HE, Reiter K, Shock A, Burmester GR, Daridon C, Dörner T; "CD22 ligation inhibits downstream B cell receptor signaling and Ca(2+) flux upon activation." **Arthritis & Rheumatology 2013 Mar; PMID: 23233360**

Sieber J, Daridon C, Fleischer SJ, Fleischer V, Hiepe F, Alexander T, Heine G, Burmester GR, Fillatreau S, Dörner T; " Active systemic lupus erythematosus is associated with a reduced cytokine production by B cells in response to TLR9 stimulation." **Arthritis Research & Therapy 2014 Nov; PMID: 25385499**

Vanessa Fleischer, Julia Sieber, Sarah J. Fleischer, Anthony Shock, Guido Heine, Capucine Daridon, Thomas Dörner; "Epratuzumab inhibits the production of proinflammatory but not regulatory cytokines by B cells from healthy donors and SLE-patients." **Arthritis Research & Therapy; under revision**

Danksagung

Zu aller erst möchte ich mich bei meinem Gruppenleiter Prof. Dr. Thomas Dörner dafür bedanken, dass er mir die Möglichkeit gab meine Doktorarbeit in seiner Arbeitsgruppe anzufertigen und mich während meiner Arbeit mit seinem Wissen, seinem Engagement und konstruktiven Diskussion unterstützt hat.

Außerdem möchte ich Dr. Capucine Daridon und Ina Kruck für ihre Hilfe und Unterstützung in den letzten Jahren bedanken, sowie meiner Arbeitsgruppe, insbesondere Karin Reiter, für die gute Zusammenarbeit und den technische Beistand sowie dem Sonderforschungsbereich sfb650 für die finanzielle Unterstützung.

Des Weiteren gilt mein Dank meiner Promotionskommission: Prof. Dr. Arturo Zychlinsky, Prof. Dr. Roland Lauster, Prof. Dr. Thomas Dörner, Prof. Dr. Andreas Radbruch und Dr. Michal Or-Guil.

Ein besonderer Dank geht an alle Blutspender und Patienten, welche mich in meiner Arbeit in den letzten Jahren tatkräftig Unterstütz haben, der FACS Abteilung, den Labormanagern und meinen Kollegen des DRFZ insbesondere Stefanie Ries und Angelika Rose, die mich mit hilfreichen Diskussion und Freundschaft emotional unterstützt haben.

Zum Schluss möchte ich mich vom ganzen Herzen bei meiner Familie und insbesondere bei Marc und Vanessa für ihre aufbauenden Worte, Liebe, Motivation und Unterstützung während der letzten Jahre bedanken.

Eidesstattliche Erklärung

Hiermit versichere ich, Sarah Jessica Fleischer, dass ich diese vorliegende Arbeit mit dem Titel **‘Dysbalanced BCR signaling in B cells of patients with systemic lupus erythematosus’** selbstständig, ohne unzulässige Hilfe Dritter und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Die eingereichte Arbeit wurde noch keinem Prüfungsamt in gleicher oder ähnlicher Form vorgelegt. Ich habe bisher an keiner in- oder ausländischen Fakultät weder ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt und besitze keinen entsprechenden Doktorgrad.

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